

2012

Molecular characterization of rainbow trout Zar1 and Zar1-like genes: their potential roles in egg quality

Chieh-Hung Lin
West Virginia University

Follow this and additional works at: <https://researchrepository.wvu.edu/etd>

Recommended Citation

Lin, Chieh-Hung, "Molecular characterization of rainbow trout Zar1 and Zar1-like genes: their potential roles in egg quality" (2012). *Graduate Theses, Dissertations, and Problem Reports*. 3518.
<https://researchrepository.wvu.edu/etd/3518>

This Thesis is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/ or on the work itself. This Thesis has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.

**Molecular characterization of rainbow trout Zar1
and Zar1-like genes: their potential roles in egg
quality**

Chieh-Hung Lin

Thesis

**Submitted to the Davis College of Agriculture, Natural
Resources, and Design
at West Virginia University
in partial fulfillment of the requirements for the degree of**

**Master of Science
in
Animal Physiology**

**Jianbo Yao, Ph.D.,
P. Brett Kenney, Ph.D.,
Marlon Knights, Ph.D.**

**Morgantown, West Virginia
2012**

**Keywords: Oocyte-specific gene, Zar1, Zar1-like, vitellogenesis,
rainbow trout**

ABSTRACT

Molecular characterization of rainbow trout *Zar1* and *Zar1*-like genes: their potential roles in egg quality

Chieh-Hung Lin

Zygote arrest 1 (*Zar1*) is a maternal-effect gene that is essential for early embryonic development. Recently, a novel gene called *Zar1*-like (*Zar1l*) was discovered. Functional studies showed that *Zar1l* plays an important role in regulating oocyte-to-embryo transition in the mouse. The objectives of this study were to characterize the rainbow trout *Zar1* and *Zar1l* genes and evaluate the potential roles of these genes in controlling egg quality in rainbow trout. Through database mining, we identified the cDNAs encoding rainbow trout *Zar1* and *Zar1l*. The *Zar1* cDNA codes for a protein of 333 aa, and the *Zar1l* cDNA encodes a protein of 323 aa. The sequences at the C terminus of the two proteins are highly conserved and contain a conserved Zinc-binding domain. Analysis of tissue distribution by RT-PCR showed *Zar1* is predominantly expressed in ovary and testis with minor expression in other somatic tissues, while *Zar1l* is exclusively expressed in ovary. The expression patterns of *Zar1* and *Zar1l* genes during early embryonic development (0h, 7.5h, 10.5h, 19.5h, 2d and 7d post fertilization) were determined by quantitative real-time PCR analysis. Both genes are highly expressed in unfertilized oocytes (0h), but they show different expression patterns. While the expression of *Zar1* gene decreases from 0h to 10.5h post fertilization, and then increases in 2d embryos, the *Zar1l* gene shows continuous reduction of expression during early embryonic development (from 0h to 2d embryos). The expression patterns of *Zar1* and *Zar1l* genes during ovarian development (pre, early, middle, and late vitellogenesis) were also determined by quantitative real-time PCR analysis. Both genes showed high expression in pre-vitellogenesis and reduced expression in early and middle vitellogenesis. In late vitellogenesis, expression of *Zar1* gene increases again, but *Zar1l* gene expression continues to decrease. To determine the role of *Zar1* and *Zar1l* genes in controlling egg quality, we analyzed the expression of both genes in eggs of different qualities (Day1, Day7, and Day14 post-ovulation). Both genes showed reduced expression in eggs of low quality. Finally, we performed a yeast-two hybridization screening to identify proteins that interact with *Zar1l* protein. Five proteins showing interactions with *Zar1l* protein were identified which include Di-N-acetylchitobiase, Serine/threonine/tyrosine-interacting protein (STYX), Ariadne-2 homolog, GH20 HexA HexB-like, and C-type mannose-binding lectin.

Acknowledgements

First of all, I would like to thank my committee members for their support and guidance. Secondly, I would like to thank my lab members for their technical help. Especially, I deeply appreciate that Dr. Yao always tolerated my mistakes and lead me to correct direction. Although I am not an outstanding student in your lab, but I hope you can be proud of me in future. Finally, I would like to thank my parents for their love and support. If they did not give me the opportunity to seek my dream, I cannot finish this degree.

Dedication

To my parents

Table of Contents

ABSTRACT	ii
Acknowledgements	iii
Dedication	iv
Introduction	1
Literature Review	3
Regulation of the oocyte-to-zygote transition	3
Maternal effect genes	5
Distribution of zygote arrest 1 (<i>Zar1</i>) and Zar1-like (<i>Zar1l</i>) in animals.....	5
Characteristics of <i>Zar1</i> and <i>Zar1l</i> proteins.....	6
Comparison of <i>Zar1</i> proteins in different species	7
Relationship between fertility and <i>Zar1</i>	7
<i>Zar1</i> and <i>Zar1l</i> expression during sexual maturation	8
<i>Zar1</i> expression during vitellogenesis, oogenesis, and embryogenesis.....	8
<i>Zar1l</i> gene	9
Other maternal effect oocyte-specific genes.....	9
Molecular characteristic of egg quality	10
Estimation or prediction of egg quality	12
The factors affecting egg quality	14
Objectives of the Study	16
Materials and Methods.....	17
Collection of samples.....	17
RNA isolation.....	17
Treatment of RNA sample with DNase	18
Reverse-Transcription Polymerase Chain Reaction and analysis	18
TA cloning.....	19
Transformation	20
Plasmid isolation	20
Double Digestion.....	21
Gel extraction.....	21
Ligation of Zar1-like gene and pGBDKT7 Vector	22
Purification of digested plasmid	22
mRNA isolation.....	22
Preparation of cDNA library	23
Yeast two hybridization	24
Yeast plasmid isolation	26
Quantitative real-time polymerase chain reaction	26
Statistical analysis.....	27
Results	28
Sequence analysis of <i>Zar1</i> and <i>Zar1l</i> genes.....	28
Tissue distribution of <i>Zar1</i> and <i>Zar1l</i> genes.....	28
Identification of proteins interacting with <i>Zar1l</i> protein.....	29
Quantitative analysis of <i>Zar1</i> and <i>Zar1l</i> gene expression.....	29
Quantitative analysis of <i>Zar1</i> and <i>Zar1l</i> gene expression in egg quality	29
Discussion	31
Tables and Figures	36
References.....	48

List of Tables

Table 1. The primers were used in experiments in this project.....	36
---------------------------------------------------------------------------	-----------

List of Figures

Figure 1. The regulation mechanism of oocyte maturation and egg activation in vertebrates.....	37
Figure 2. The comparison of the sequence of <i>Zar1</i> and <i>Zar1l</i> gene in rainbow trout.....	38
Figure 3. <i>Zar1</i> and <i>Zar1l</i> protein sequences	39
Figure 4. Comparison between the amino acid sequences of <i>Zar1</i> and <i>Zar1l</i> protein	40
Figure 5. Amino acid identity of <i>Zar1</i> and <i>Zar1l</i> protein between the different specie.....	41
Figure 6. Neighbor joining phylogenetic trees of <i>Zar1</i> and <i>Zar1l</i> proteins.	42
Figure 7. RT-PCR analysis of tissue distribution of <i>Zar1</i> and <i>Zar1l</i> genes...	43
Figure 8. The Yeast-two hybridization system for protein-protein interaction of <i>Zar1l</i> gene.....	44
Figure 9. Real-time PCR analysis of expression levels of <i>Zar1</i> and <i>Zar1l</i> genes in ovarian stages.....	45
Figure 10. Real-time PCR analysis of expression levels of <i>Zar1</i> and <i>Zar1l</i> genes in embryonic development.....	46
Figure 11. Real-time PCR analysis of expression levels of <i>Zar1</i> and <i>Zar1l</i> genes in egg quality.....	47

Introduction

Rainbow trout is one of the most important cold-water fish species in the USA due to its importance for food production, sport fisheries and as a research model. The egg of rainbow trout is especially important because it is one of the determining factors of embryo quality. Egg quality can be highly variable, partly because it involves multiple developmental phases of oocyte maturation, vitellogenesis, and competent egg ovulation in which maternal RNA transcripts and proteins play an important role (Thorgaard, Bailey et al. 2002).

Zygote arrest 1 (*Zar1*) gene was the first oocyte-specific maternal-effect gene to be identified, and it plays an essential role during the oocyte-to-embryo transition in mice (Wu, Viveiros et al. 2003). Since its discovery, the gene has been studied in several species, including human, frog, cattle, pig, chicken and zebrafish (Brevini, Cillo et al. 2004; Uzbekova, Roy-Sabau et al. 2006; Sangiorgio, Strumbo et al. 2008; Michailidis, Argiriou et al. 2010). These studies found that *Zar1* gene is expressed in distinct tissues in different species, but it is always expressed in gonad tissues in each species. For example, expression of *Zar1* gene is detected in ovary, oviduct, kidney, spleen, brain, liver, testes, and epididymis of the chicken (Michailidis, Argiriou et al. 2010), but it is only expressed in ovary and testis in the human (Wu, Viveiros et al. 2003). In chickens, the level of ovarian *Zar1* mRNA is up-regulated during sexual maturation, but drops significantly in old birds (Michailidis, Argiriou et al. 2010). In humans, *Zar1* mRNA is found in one- and two-cell stage embryos, but not in four- to eight-cell embryos and the blastocyst (Wu, Viveiros et al. 2003). Furthermore, the similarity of human *Zar1* protein with its cattle, mouse, frog, zebrafish, and pufferfish counterparts is 77.5%, 88.1%, 76.6%, 66.7%, and 70.2%, respectively (Brevini, Cillo et al. 2004).

Zar1l gene is a novel gene, and it was first identified in 2008 (Sangiorgio, Strumbo et al. 2008). The structure is similar to *Zar1* gene, but they are located on different chromosomes. *Zar1l* gene is predominantly expressed in oocytes and early preimplantation embryos (Sangiorgio, Strumbo et al. 2008). In chickens, its expression is up-regulated during sexual maturation (Michailidis, Argiriou et al. 2010).

Literature Review

Regulation of the oocyte-to-zygote transition

The fertilized oocyte remodel to a totipotent zygote, and this transition occurs without transcription, but depends on the mRNAs accumulated in the oocyte during oogenesis (Stitzel and Seydoux 2007). During oocyte maturation, the oocytes arrested in prophase of meiosis I enter meiotic M phase and initiate the meiotic divisions by the stimulation of extracellular signals (Kishimoto 2003). Additionally, the oocytes are ovulated and become capable of fertilization before reaching a second arrest point. The egg activation is triggered by sperm entry, and finishes the transformation to a zygote by signaling the completion of meiosis, the formation of pronuclei, and the first mitotic division (Malcuit, Kurokawa et al. 2006).

Oocyte maturation needs the synthesis of new proteins produced in the correct succession; timing is ensured by the interdependent translation activation events (Vasudevan, Seli et al. 2006). For instance, the translation of the cyclin-dependent kinase (CDK)-binding protein RINGO/Spy activates maturation promoting factor (MPF) during oocyte maturation in *Xenopus*. After activation, MPF stimulates the translation of proteins required to maintain metaphase II arrest in the matured oocyte (Fig. 1);(Keady, Kuo et al. 2007). During egg activation, additional RNAs are recruited for translation. Moreover, the oocyte polysomes are enriched for transcripts involved in cell homeostasis, and the zygotic polysomes are enhanced for transcripts involved in macromolecular biosynthesis (Stitzel and Seydoux 2007). The oocyte mRNAs are activated for translation by freeing the mRNAs from complexes that block translation initiation (Tadros and Lipshitz 2005; Vasudevan, Seli et al. 2006).

Oocyte maturation and egg activation also can stimulate mRNA degradation,

and there are about 15 % of transcripts degraded during maturation in mice. The degradation is selective and has priority to remove transcripts for prophase arrest and oocyte maturation (Wang, Piotrowska et al. 2004). Further degradation appears after fertilization, and it guides the transition to zygotic control (Hamatani, Carter et al. 2004; Tadros and Lipshitz 2005; Evsikov, Graber et al. 2006). Proteins degradation has also been discovered during the oocyte-to-zygote transition, and the components of the ubiquitin-proteasome pathway are well represented in the oocyte transcriptome (Evsikov, Graber et al. 2006). Protein degradation often occurs to inactivate proteins that are required early in the transition but that would be damaging later (Mendez, Barnard et al. 2002). Protein degradation can also restrict proteins to specific areas of the eggs (DeRenzo and Seydoux 2004). So protein degradation can be a way to partially clean the germline program of the egg to promote totipotency in the zygote (Stitzel and Seydoux 2007).

The changes during the oocyte-to-zygote transition are not only confined to proteins and RNAs but also influence cellular organelles. For instance, the meiotic divisions make many changes in the oocyte nucleus, and the organelles in the cytoplasm and at the cell periphery are also affected (Fig. 1) (FitzHarris, Marangos et al. 2003; Stitzel and Seydoux 2007). These changes are initiated by extracellular ligands in oocyte maturation and by Ca^{2+} signaling inside the egg in egg activation (Voronina and Wessel 2003; Malcuit, Kurokawa et al. 2006). After the triggers, the cell cycle machinery can be as an internal master timer for driving and coordinating the transition. Our understanding of the signaling and remodeling events for oocyte-to-zygote transition has increased, and more definitive gauges can be provided to identify the elements of a healthy egg (Stitzel and Seydoux 2007).

Maternal effect genes

Maternal effect is associated with the maternal products during the embryonic development. The maternal products include maternal proteins and mRNAs that are recruited and accumulated during oocyte maturation or following fertilization. In addition, the maternal effect depends on maternal mRNA, because the fully grown oocyte and the one-cell embryo are transcriptionally silent but translationally active. The maternal-to-zygotic transition happens during the late one-cell or early two-cell stage (Ma, Zeng et al. 2006). Furthermore, several maternal transcripts and proteins affect the initial divisions during embryonic development before embryonic genome activation (Latham 1999; Latham and Schultz 2001; Schultz 2002). The activation of the embryonic genome is a necessary event in early development after the fertilization, and the maternal products can create appropriate conditions for activation of embryonic genome (Tong, Bondy et al. 2002). In genetic assay, the maternal-effect gene mutation females will produce the mutant offspring, and the homozygous-null genotype of mutant litters depend on the genotype of mother (Griffiths AJF 2000). Moreover, several studies showed that knockout of maternal-effect genes does not affect oocyte development, maturation, and ovulation, but impairs embryonic development (Christians, Davis et al. 2000; Tong, Gold et al. 2000; Burns, Viveiros et al. 2003; Payer, Saitou et al. 2003; Wu, Viveiros et al. 2003).

Distribution of zygote arrest 1 (*Zar1*) and Zar1-like (*Zar1l*) in animals

Zar1 gene expression was first detected in ovaries in the mouse (Wu, Viveiros et al. 2003). In *Xenopus laevis*, *Zar1* expression can be found in ovaries, muscle, and lung tissues but not in testis (Wu, Wang et al. 2003). In cattle, *Zar1* gene is expressed in ovary, testis, skeletal muscle, brain, pituitary and myocardium, while *Zar1l*

transcripts is present in ovary, oviduct, testis, kidney, lung, and stomach (Brevini, Cillo et al. 2004; Uzbekova, Roy-Sabau et al. 2006). Pig *Zar1* gene transcripts can be found in ovary, testis, brain, and pituitary. Furthermore, both chicken *Zar1* and *Zar1l* transcripts are detected in the ovary, oviduct, testes, and epididymis, but *Zar1* transcripts are present at very low levels in the kidney, spleen, brain, and liver (Michailidis, Argiriou et al. 2010).

Characteristics of *Zar1* and *Zar1l* proteins

There are some oocyte-specific genes called maternal effect genes that can affect early cleavage regulation (Dean 2002). *Zar1* gene is one of the maternal effect genes. Human *Zar1* gene is located on chromosome 4 in a region of conserved synteny to that on mouse chromosome 5. The gene spans 4.1 kb and contains four exons. The encoded proteins are 361 amino acids in mouse and 424 amino acids in human. The proteins of human and mouse *Zar1* share 59% amino acid identity and both have an atypical plant homeo domain (PHD) motif which is located at the C termini of *Zar1* protein (Wu, Viveiros et al. 2003). A typical PHD motif is C₄-H-C₃ (C-X₂-C-X₍₉₋₂₁₎-C-X₍₂₋₄₎-CX₍₄₋₅₎-H-X₂-C-X₍₁₂₋₄₆₎-C-X₂-C) zinc-binding amino acid arrangement, and the PHD motif of *Zar1* is a C₈ pattern (C-X₂-C-X₁₃-C-X₂-C-X₄-C-X₁-C-X₁₇-C-X₂-C) (Wu, Viveiros et al. 2003). However, atypical PHD motif in *Zar1* and *Zar1l* orthologs is C₁₂ pattern (C-X₂-C-X₁₀-C-X₁₂-C-X₂-C-X₁₃-C-X₂-C-X₄-C-X₁-C-X₁₇-C-X₂-C-X₆-C) (Aasland R. 1995). A substitution of C-to-H is also discovered in the disease associated PHD containing protein ATRX (Gibbons, Bachoo et al. 1997; Wu, Viveiros et al. 2003). Mutagenesis of C to H in PHD containing protein KAP-1 has little effect, suggesting that these amino acids are functionally similar (Capili, Schultz et al. 2001). PHD domains can be found in two major kinds of proteins that

are transcriptional activators, repressors, or cofactors and subunits of complexes that modulate chromatin. Therefore, *Zar1* protein may function as a transcriptional regulator, and the C terminus of *Zar1* protein might be functionally important (Wu, Viveiros et al. 2003).

Comparison of *Zar1* proteins in different species

The length of *Zar1* protein is different in diverse species, including human, mouse, frog, cattle, pig, chicken, zebrafish, and pufferfish. The human *Zar1* protein (424 amino acids) is the longest, and the frog *Zar1* protein is 295 amino acids in length. The length of mouse *Zar1* protein is 361 amino acids. Zebrafish and pufferfish *Zar1* genes encode proteins of 329 and 320 amino acids, respectively (Wu, Wang et al. 2003). The bovine *Zar1* protein is 384 amino acids and the pig gene encodes a protein of 387 amino acids (Uzbekova, Roy-Sabau et al. 2006). Chicken *Zar1* gene encodes a shorter protein of 220 amino acids (Michailidis, Argiriou et al. 2010). The similarity of human *Zar1* with cattle, mouse, frog, zebrafish, and pufferfish orthologs are 77.5%, 88.1%, 76.6%, 66.7%, and 70.2%, respectively (Brevini, Cillo et al. 2004). Furthermore, the sequence identity of human *Zar1* protein with mouse, frog, pig, cattle, zebrafish, and pufferfish *Zar1* proteins at the C-termini are 91.3%, 97.1%, 96%, 96%, 89.3%, and 83.5%, respectively (Wu, Wang et al. 2003; Uzbekova, Roy-Sabau et al. 2006).

Relationship between fertility and *Zar1*

Wu et al (2003) reported that *Zar1*^{+/-} and *Zar1*^{-/-} male and female mice did not show any histological abnormalities. The *Zar1*^{-/-} males were fertile. Offspring were produced when *Zar1*^{+/-} females mate with *Zar1*^{+/-} males, but no litters were produced

when *Zar1*^{-/-} females bred with *Zar1*^{+/-} males. Because most embryos of *Zar1*^{-/-} females cannot develop to two-cell stage, *Zar1*^{-/-} females are sterile. Therefore, *Zar1* is essential for female fertility (Wu, Wang et al. 2003).

***Zar1* and *Zar1l* expression during sexual maturation**

Zar1 gene expression significantly increases in ovary during sexual maturation, and *Zar1* and *Zar1l* gene expression is related to the production of mature spermatozoa from sexually mature males. The sexually mature chicken (52 weeks old) had significantly higher levels of *Zar1* mRNA than pre-pubertal chickens, but *Zar1* mRNA abundance in old birds (104 weeks old) is very low. Moreover, there is a significant up regulation of both *Zar1* and *Zar1l* genes in the testes from immature to mature roosters. The levels of both *Zar1* and *Zar1l* genes in the testes of low fertility roosters (72 weeks old) are still high, but significantly lower than that of mature roosters (52 weeks old) (Michailidis, Argiriou et al. 2010).

***Zar1* expression during vitellogenesis, oogenesis, and embryogenesis**

Zar1 mRNA expression decreases and disappears during embryogenesis in various species. Mouse *Zar1* expression decreases in normal two-cell embryos that is associated with the rapid degradation of maternal transcripts after fertilization (Flach, Johnson et al. 1982). High levels of *Zar1* transcript expression in the oocytes within ovaries were demonstrated by in situ hybridization and immunohistochemistry, and *Zar1* expression in oocytes can be detected from primary follicle stage through the antral follicle stage. However, *Zar1* expression cannot be found at the primordial follicle stage (Wu, Viveiros et al. 2003). Frog *Zar1* mRNA expression decreases dramatically during the neurula stage and disappears in the tadpole-stage embryos

(Wu, Wang et al. 2003). In addition, bovine *Zar1* expression is detected in oocyte and early embryos from 2-cell to blastocyst, and *Zar1* mRNA significantly increases at 4-cell stage (Brevini, Cillo et al. 2004). Pig *Zar1* mRNA decreases from zygote to eight-cell stages, and shows very low expression at morula and blastocyst stages (Uzbekova, Roy-Sabau et al. 2006). Furthermore, human *Zar1* mRNA is present at one- and two-cell stage embryos, but disappears from four-, eight-cell embryos and blastocyst (Wu, Viveiros et al. 2003). Chicken *Zar1* and *Zar1l* gene expression can be observed from oocytes to embryos at every stage (Michailidis, Argiriou et al. 2010).

***Zar1l* gene**

A cDNA encoding a protein structurally associated with *Zar1* was discovered recently. This protein is well conserved among the vertebrate lineage. The structure of the new gene is similar with *Zar1* gene, and they are located on different chromosomes. Thus this novel gene was named *Zar1l* (Sangiorgio, Strumbo et al. 2008). For example, the structure of the chicken *Zar1* and *Zar1l* genes are similar but distinct. The homology of C-terminal domain of *Zar1* and *Zar1l* proteins in chicken is approximately 74%, and *Zar1l* also contains an atypical PHD motif. The information suggests that *Zar1l* is also involved in translational regulation events during the oocyte-to-embryo transition (Michailidis, Argiriou et al. 2010). Microinjection of mutant *Zar1l* protein into embryo induces embryonic arrest at the two cell stage in mice (Hu, Wang et al. 2010).

Other maternal effect oocyte-specific genes

Maternal effect oocyte-specific genes such as *Mater* or *Nalp5*, *Nalp9*, *Zar1*, *Hsfl*, *Dnmt1*, *Brg1*, and *Nmp2* have been identified in many vertebrate species (Michailidis, Kalivas et al. 2009). For instance, *Mater* is a single copy gene expressed

exclusively in oocytes, and its transcripts are accumulated during oogenesis, but do not appear in early embryos. Mater has been identified as an oocyte-specific autoantigen associated with ovarian autoimmunity (Tong and Nelson 1999). Mater-null females have normal fertilization, and their zygotes can progress through first cleavage and are arrested at 2-cell stage of the subsequent development. Initiation of the transcription-translation machinery is not affected in early embryos lacking Mater (Tong, Gold et al. 2000). In addition, the abundance of Mater mRNA is relatively greater in the ovaries of younger woman. The human Mater is present within the oocytes but not in the follicular cells, and the expression of Mater gene is down regulated significantly during oocyte maturation and after 8-cell stage in sheep (Tong, Bondy et al. 2002; Bebbere, Bogliolo et al. 2008). Heat-shock factor-1 (Hsf1) is a major trans-activator of stress-inducible genes in response to environmental changes. Hsf1 also relates to extra-embryonic development and female fertility in mice. Mouse embryos lacking Hsf1 proteins in their mothers cannot develop properly beyond the zygotic stage, but the oocytes can still ovulate and be fertilized normally (Christians, Davis et al. 2000). Stella is another maternal effect gene, which is primarily expressed in primordial germ cells, oocytes, pre-implantation embryos, and pluripotent cells. Females deficient in Stella have reduced fertility due to lack of maternally inherited Stella protein in their oocytes, and embryos without Stella are compromised in pre-implantation development and scarcely reach the blastocyst stage (Payer, Saitou et al. 2003). Basonuclin is another known maternal effect gene expressed in oocytes and one-cell embryo. Basonuclin-deficient oocytes have increased failure of oocyte development and early embryo (Ma, Zeng et al. 2006).

Molecular characteristic of egg quality

Egg quality which is one of the limiting factors for reproductive success can be defined as the ability of the egg to be fertilized and to subsequently develop into a normal embryo (Bobe and Labbé 2010). Normally, the unfertilized egg or female gamete is an oocyte arrested in metaphase of the second meiotic division, and the metaphase oocyte is the final product of the oogenesis (Tata 1986). The egg components accumulated during oogenesis play a key role in the coordinated assembly of a good quality oocyte. When the oocyte is fertilized, it will develop into a normal embryo. An egg can increase in volume from massive incorporation of yolk proteins during vitellogenesis (Wallace and Selman 1981; Wallace and Selman 1985; Brooks, Tyler et al. 1997; Patiño and Sullivan 2002; Mommsen 2008). The yolk protein of ovulated oocyte contains many components such as maternal mRNAs, proteins, vitamins and hormones (Tata 1986; Howley and Ho 2000; Pelegri 2003). After fertilization, maternal factors support early embryonic development until the activation of zygotic transcription that occurs during maternal-embryo transition. The maternal-embryo transition usually appears at the mid-blastula stage in fish or other lower vertebrates, so it is also called mid-blastula transition (Kane and Kimmel 1993). When the maternal mRNA translation in rainbow trout eggs is inhibited by cycloheximide, embryonic cleavage is delayed (Nagler 2000). Furthermore, storage and localization in the oocyte affect the function of maternal factors in developing embryo. Recently, molecular analyses show that some maternal mRNA expressed in different abundance in eggs of varying quality, and the spatial distribution of specific maternal mRNAs within the oocyte plays a key role for specifying the dorso/ventral axis of the embryo (Bally-Cuif, Schatz et al. 1998; Howley and Ho 2000; Aegerter, Jalabert et al. 2005; Bonnet, Fostier et al. 2007). For instance, the mRNA level of nucleoplasmin (npm2) which is a maternal-effect gene decreases during the egg post-

ovulatory ageing in rainbow trout (*Oncorhynchus mykiss*), and the egg quality also progressively decreases (Burns, Viveiros et al. 2003; McLay and Clarke 2003; Aegerter, Jalabert et al. 2005). Similarly, prohibitin 2 (phb2) mRNA expression was also observed to be associated with eggs of varying quality in rainbow trout (Bonnet, Fostier et al. 2007).

Estimation or prediction of egg quality

The quality of an egg can be defined as its ability to be fertilized, and subsequently develop into a normal embryo. Before fertilization, the size of unfertilized egg can temporarily be used to estimate the overall developmental potential of the egg, but small eggs produce similar rates of fertilization than larger ones in rainbow trout under normal conditions of temperature, post-ovulatory ageing, and husbandry practices (Bromage, Jones et al. 1992; Bobe and Labbé 2010). In addition, the appearance or morphology of unfertilized egg can also be used to estimate the developmental potential of the egg, but this estimation is limited under normal hatchery conditions and lacks a consistent relationship between distribution of lipid droplets and egg quality in hatchery-raised rainbow trout (Ciereszko, Wojtczak et al. 2009). Furthermore, there are some indirect measurements such as physico-chemical parameters of ovarian or coelomic fluid in hatched eggs to predict egg quality. Several studies showed that the low pH of coelomic or ovarian fluid induces the reduced egg quality. In turbot (*Scophthalmus maximus*) and rainbow trout, the drop of egg quality during egg post-ovulatory ageing is related to the drop of ovarian fluid pH (Fauvel 1993; Lahnsteiner 2000; Aegerter and Jalabert 2004). The vitellogenin fragments, originating from the eggs, accumulate in coelomic fluid during post-ovulatory ageing, and the coelomic fluid pH reduces during the post-ovulatory ageing

because of the presence of egg content in the fluid. In addition, the broken eggs of rainbow trout can decrease the pH of coelomic fluid (Rime, Guitton et al. 2004; Dietrich, Wojtczak et al. 2007). However, the pH variation range during the post-ovulatory ageing is limited, and there is no significant linear regression between pH and embryonic survival (Lahnsteiner 2000; Aegerter and Jalabert 2004).

Fertilization success is one of the earliest estimators and the key components of egg quality, but opaque eggs from some species are not easy to detect. For instance, the eggs from rainbow trout are necessary to be fixed and stained with specific dyes (Bobe and Labbé 2010). However, fertilization rate cannot reflect further developmental success in several marine species (Shields, Brown et al. 1997). The embryonic division patterns can also be used to measure the egg quality for some species, and abnormal cleavage and blastomere morphology affect the early embryonic mortalities and developmental success at late stages respectively (Shields, Brown et al. 1997; Kjørsvik, Hoehne-Reitan et al. 2003; Avery and Brown 2005). Finally buoyancy of pelagic eggs is one way to predict the ability of normal development (Sakai 1985).

Successful development of fertilized eggs can be characterized by the embryonic survival at a specific embryonic step, such as eyed, hatching, and yolk-sac resorption stages. Moreover, monitoring the survival at successive developmental steps can characterize the timing of embryonic mortalities by different experimental treatments (Kopeika, Kopeika et al. 2003; Bonnet, Fostier et al. 2007).

Embryonic or larval malformation is another valuable tool to characterize developmental potential of fertilized eggs. For instance, specific types of malformations in offspring of rainbow trout were induced by specific breeding conditions of brood fish. The “cyclop” malformation has high chance to appear at the

long-term post-ovulatory ageing of the eggs (Aegerter, Jalabert et al. 2004; Bonnet, Fostier et al. 2007). In addition, larval malformation is associated the sperm damage in some species, but the effect does not show up in the rainbow trout (Horvath and Urbányi 2000; Labbe, Martoriati et al. 2001). In many cases, sperm DNA defects can be repaired by the egg repair system after fertilization (Kopeika, Kopeika et al. 2004).

The factors affecting egg quality

Many factors have been studied and shown to affect the egg quality. Broodstock nutrition is one of the important factors affecting not only egg quality but also fecundity and gametogenesis, such as spawn quality, spawn frequency, eicosanoid production, steroid hormone levels, and gonadotropin-induced maturation (Kjørsvik, Mangor-Jensen et al. 1990; Brooks, Tyler et al. 1997; Izquierdo, Fernandez-Palacios et al. 2001). In addition, some components of broodstock diet can affect embryonic development. For instance, suboptimal levels of vitamin E reduce larval survival, and developmental abnormalities increase. Vitamin A is important for embryo and larval development (Izquierdo, Fernandez-Palacios et al. 2001; Palace and Werner 2006). In rainbow trout, suboptimal levels of ascorbic acid in the diet can result in low embryonic survival at the eyed stage (Blom and Dabrowski 1995). Moreover, the essential fatty acids are necessary to assure normal embryonic and larval development (Leray, Nonnotte et al. 1985). According to these studies, deficiencies in essential components of the diet will damage broodfish fecundity, egg quality and embryo development.

Temperature is a major environment factor influencing egg quality, and both high and low temperature can have negative impacts on egg quality during the reproductive season and spawning. For example, egg quality of rainbow trout is

reduced at temperatures of 15 °C and above (Pankhurst, Purser et al. 1996; Aegerter and Jalabert 2004). Another environment factor is photoperiod, and available data show that photoperiod-induced manipulation of spawning can reduce egg quality. In addition, the negative impact on egg quality is highly associated with type of photoperiod regime. Suboptimal temperature around spawning time will further increase any negative effect of the photoperiod regime on egg quality (Bobe and Labbé 2010). For instance, a significant decrease of egg quality of rainbow trout occurs in advanced spawning by artificial photoperiod from June to July, even though the water temperature is kept at 12 °C during the reproductive season (Bonnet, Fostier et al. 2007). The last environment factor is salinity, based on observation that some species spend a part of their life in sea and then migrate to fresh water for reproduction. Water salinity becomes one key effect for reproductive success during the reproductive season. Atlantic salmon females kept in sea during the reproductive season would delay or block ovulation (Haffray, Fostier et al. 1995).

There are other factors affecting egg quality, such as husbandry practices, stress, exposure to xenobiotics and pollutants, physiochemical properties of the water, and genetic differences. Husbandry practices include spawning incubation, egg post-ovulation ageing, and gamete handling post-stripping (Brooks, Tyler et al. 1997; Stoddard, Parsons et al. 2006; Bobe and Labbé 2010). Little information is available regarding the effect of ongoing genetic selection on egg quality. For example, a decrease in fertility in farmed animals is associated with genetic selection for non-reproductive phenotypes, so genetic selection is a direction of investigations for aquaculture in the future. The predictive estimators or markers of egg quality can primarily be applied in the field or in the industry, which would prevent the risk of mixing egg batches of poor and good quality (Weigel 2006; Bobe and Labbé 2010).

Objectives of the Study

Zar1 and *Zar1l* genes have been studied in several mammalian species and their roles in controlling early embryogenesis have been documented. However, in fish, studies of these important maternal effect genes are limited. Specifically, the *Zar1l* gene is still a novel gene, and limited information about this gene is available. Thus the objectives of this study were: 1) to clone and characterize the expression of *Zar1* and *Zar1l* genes in rainbow trout, 2) to identify other proteins that interact with *Zar1l* protein, and 3) To determine the expression of *Zar1* and *Zar1l* genes in eggs of various qualities.

Materials and Methods

Collection of samples

Rainbow trout tissues including gill, liver, stomach, kidney, ovary, testis, skin, heart, brain, and spleen were obtained from USDA NCCCWA. These tissue samples for RT-PCR were quickly frozen in liquid nitrogen and stored in -80°C until use. The ovary stages were characterized by the size of the oocytes as follows: early pre-vitellogenesis (0.5mm), late pre-vitellogenesis (0.65mm), early-vitellogenesis (0.65-1.1mm), mid-vitellogenesis (1.1-2.1mm) and late-vitellogenesis (2.1-4.0mm). Embryonic samples were collected at 0 hour, 7.5 hours, 10.5 hours, 19.5 hours, 2 days and 7 days after fertilization. Egg samples from females on day 1, day 7, and day 14 post-ovulation (D1PO, D7PO and D14PO) were collected. The embryonic survival rates of these egg samples (D1PO, D7PO, and D14PO) were determined to be 95%, 75% and 35%, respectively. For each egg quality sample, eggs from different females were mixed (8 eggs per sample, n=4). All samples were quickly frozen in liquid nitrogen and stored in -80°C until use.

RNA isolation

Total RNA of each tissue (or eggs) was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Before homogenizing, the blade of the homogenizer was washed three times in beakers containing MilliQ water. Frozen tissue (0.3 g) was placed in 3 ml of Trizol solution, and kept on ice. The tissue was homogenized several times until the tissue was completely homogenized. After homogenizing, the homogenous solution was incubated at room temperature for 5 minutes, and then 0.8 ml of chloroform was added into the homogenous solution. The mixture was shaken vigorously for at least

15 seconds by hand and left at room temperature for 5 minutes. After 5 minutes, the mixture was centrifuged at 10,500 rpm for 15 minutes at 4°C, and the upper aqueous phase was transferred to a new 1.5-ml tube. 2 ml of isopropanol was added to the tube, and the mixture was mixed by shaking and incubated at room temperature for 10 minutes. The tube was then centrifuged at 10,500 rpm for 10 minutes at 4 °C, and the supernatant was poured off. The pellet was dried briefly by placing the tube upside down on Kim Wipers, and then 4 ml of 4M Lithium Chloride was added into tube and kept on ice for 30 minutes. During the 30 minutes, the pellet was resuspended by vortexing or pipetting. The sample was centrifuged at 14,000 rpm at 4°C for 20 minutes, and the Lithium Chloride was poured off. Then 1 ml of cold 75% ethanol was added to wash the pellet, and the pellet was air dried for 5-10 minutes after spinning down and pouring off ethanol. Finally, the pellet was resuspended in 30 µl of RNase-free water. The RNA concentration was measured by NanaDrop and stored in -80°C until use.

Treatment of RNA sample with DNase

Total RNA was treated with DNase by mixing 3 µl of total RNA (2 µg/µl), 3 µl of 10x DNase I buffer, 23 µl of H₂O, and 1 µl of DNase I (Ambion) in a tube. The mixture was incubated at a 37 °C waterbath for 25 minutes. After incubation, 5 µl of resuspended DNase Inactivation Reagent was added into the tube and mixed well, and then the mixture was incubated for 2 minutes at room temperature. Finally, the sample was centrifuged at 12,000 rpm for 1.5 minutes and the supernatant containing DNase treated RNA was collected.

Reverse-Transcription Polymerase Chain Reaction and analysis

8 µl of DNase treated RNA was transferred into a PCR tube and 1 µl of Oligo(dT)₁₈ (500 µg/ml), 1 µl of dNTP mix (10 mM), and 3 µl of H₂O, were added. The mixture was then heated to 65 °C for 5 minutes in a PCR machine and quickly chilled on ice for 2 minutes. 4 µl of 5X first-strand buffer, 2 µl of 0.1 M DTT, and 1 µl of SuperScriptTM III RT (200 U/µl, Invitrogen) were added to the tube. The contents were mixed by pipetting and incubated at 42 °C for 50 minutes in a PCR machine. The reaction was inactivated by heating at 70 °C for 10 minutes. The cDNA product was diluted by adding 80 µl of H₂O, and kept on ice.

The cDNA of each tissue was amplified with specific primers by PCR. Two µl of cDNA was combined with 7 µl of PCR mix (containing 5X PCR buffer, 10 mM dNTP, and 25 mM MgCl₂), 1.25 µl of 10 µM specific forward primers, 1.25 µl of 10 µM specific reverse primers (Table 1), 0.25 µl of Tag polymerase (5 U/µl), and 13.25 µl of water. After preparation of PCR mixture, the samples were placed in a PCR machine, and PCR was performed using the following protocol. The samples were denatured at 95 °C for 3 minutes, followed by 35 cycles including denaturing at 95 °C for 40 seconds, annealing at 58 °C for 45 seconds, and extension at 72 °C for 1 minute. Finally, the products were incubated at 72 °C for additional 10 minutes and maintained at 4 °C. Water was used to replace cDNA samples as a negative control. The β-actin gene was used as the cDNA quality control, and *Zar1* and *Zar1l* gene specific primers were used to amplify these genes. After amplification, the PCR product was detected by electrophoresis with 1% agarose gel.

TA cloning

Cloning of PCR products was performed using the TA cloning kit from Promega according to the kit manual. Three µl of PCR product (*Zar1* or *Zar1l*) was

add into a 1.5 ml tube which contains 5 µl of 2X Rapid Ligation Buffer, 1 µl of pGEM[®]-T Easy Vector (50ng), and 1 µl of T4 DNA Ligase (3 Weiss units/µl). The mixture was mixed well by pipetting, and then incubated at room temperature for 1 hour.

Transformation

1 µl of TA cloning ligation mixture was added into 25 µl of competent cells (Novagen) kept on ice. After brief mixing, the mixture was kept on ice for 5 minutes. The tube was placed in a 42°C water bath for exactly 30 seconds and then kept on ice for 2 minutes followed by addition of 80 µl of room temperature SOC medium. Finally, the cells were cultured on selecting LB plate, and the plate was incubated at 37 °C overnight.

Plasmid isolation

One colony was transferred to 3 ml of Ampicillin or Kanamycin LB broth, and the culture was incubated at 37 °C with 250 rpm shaking overnight. Plasmid was isolated from bacteria using the QIAprep Spin Miniprep Kit (QIAGEN). After overnight culture, the 3 ml of cells were centrifuged to get the pellet, and the pellet was resuspended in 250 µl of Buffer P1. 250 µl of Buffer P2 was added to the mixture and mixed by inverting 4-6 times. Then 350 µl of Buffer N3 was added to the sample, and the mixture was mixed well by inverting 4-6 times. The supernatant was obtained by centrifugation at 13,000 rpm for 10 minutes, and it was applied to the QIAprep spin column. After 1 minute centrifugation, the flow-through was discarded, and 0.5 ml of Buffer PB was used to wash the column. The flow-through was discarded after 1 minute centrifugation, and then the column was washed by 0.75 ml of Buffer PE

and centrifuged for 1 minute. After 1 minute centrifugation, the QIAprep column was placed in a new 1.5 ml tube, and 30 µl of water was added at the center of the column to elute the plasmid. The plasmid was collected by centrifugation for 1 minute.

Double Digestion

The *Zar1l* cDNA clone and the pGBDKT7 vector were digested by BamHI and NdeI (New England BioLabs). 5 µl of *Zar1l* cDNA or vector plasmid were mixed with 2 µl of 10X NEBuffer, 0.2 µl of 100X BSA, 0.5 µl of BamHI, 0.5 µl of NdeI, and 11.8 µl of water in a tube. The mixture was incubated in a PCR machine at 37 °C overnight.

Gel extraction

Agarose gels containing the DNA fragments were collected and DNA from the gels was isolated using a QIAGEN kit. At the beginning, the empty tube and gel slice were weighed and then 3 volumes of QG Buffer were added to 1 volume of gel (100 mg ~ 100 µl). The tube was incubated at 50 °C for 10 minutes, and the mixture was vortexed every 2 minutes during the incubation help the gel dissolve. When the gel was dissolved completely, 1 gel volume of isopropanol was added and mixed well. Then the mixture was transferred to a QIAquick spin column and centrifuged for 1 minute. 0.5 ml of Buffer QG was used to wash the column and the QIAquick spin column was washed with 0.75 ml of Buffer PE, and the flow-through was removed by 1 minute centrifugation. After additional 1 minute centrifugation, the QIAquick spin column was placed on a clean 1.5 ml tube, and 30 µl of water was added to the center of the QIAquick membrane. The DNA fragment was eluted by 1 minute centrifugation.

Ligation of Zar1-like gene and pGBDKT7 Vector

2 µl of pGBDKT7 Vector and 6 µl of *Zar1l* fragments were placed in a 1.5 ml tube. Then 1 µl of 10X T4 DNA ligase buffer and 1 µl of T4 DNA ligase were added to the tube and mixed. The sample was incubated at room temperature for 3 hour. The ligation product was transformed to competent cells, and they were cultured on Kanamycin LB plate.

Purification of digested plasmid

The TA cloning plasmid of specific gene (*Zar1* and *Zar1l*) was digested for T7 or Sp6 promoter. T7 promoter was digested by SacII, and Sp6 promoter was by SacI. The 50 µl of digested plasmid was added to 50 µl of the water, and then 100 µl of phenol-chloroform (25:24:1). The mixture was centrifuged at the max speed for 5 minutes, and there are two phases in the tube. The upper phase was transferred to a clean tube, and one ninth of upper phase of 3M NaAc was added to the tube. Before the mixture was incubated at -80 °C for one hour, the 2.5 time of the mixture of ethanol was loaded to the sample. When the mixture was taken from the freezer, the sample was centrifuged at max speed for 15 minutes. Then the pellet was washed by 75 % ethanol, and it was resuspend in 10 µl of water.

mRNA isolation

A 0.1 mg of high quality total RNA was added to 500 µl of RNase-free water in sterile tube, and the sample was heated at 65 °C for 10 minutes. Then 3 µl of Biotinylated-Oligo(dT) Probe and 13µl of 20X SSC was brought to the sample, and the sample was incubated at room temperature until completely cooled. The SA-

PMPs should be resuspended by flicking the tube, and it was captured by putting at the Magnetic Stand. After removing the supernatant, the SA-PMPs were washed with 0.5X SSC three times. Each wash should use the Magnetic Stand to remove supernatant carefully. The washed SA-PMPs were resuspended in 100 μ l of 0.5X SSC, and then the entire annealing RNA was added to the SA-PMPs. Before the mixture was incubated at room temperature for 10 minutes, the mixture should be mix well by inversion. When the tube was put at Magnetic Stand, the supernatant could be carefully removed. The particles were washed with 0.1X SSC four times, and the step should cooperate with the Magnetic Stand. Then the mRNA was eluted in 100 μ l of RNase-free water by resuspending the particles and waiting for 1 minute. When the particles were captured by Magnetic Stand, the eluted mRNA could be transferred to a sterile tube. After an additional elution for 150 μ l of mRNA, the final collection should be 250 μ l. (Promega)

Preparation of cDNA library

2 μ l of mRNA sample was combined with 1 μ l of CDS III (Olig-dT Primer) and 1 μ l of deionized water, and the mixture was incubated at 72 °C for 2 minutes. After heating, the sample was cooled on ice for 2 minutes, and then it was centrifuged at 14,000 g for 10 seconds. The sample was added to the reaction mix which contained 2 μ l of 5X First-Strand Buffer, 1 μ l of DTT (100 mM), 1 μ l of dNTP mix (10 mM), and 1 μ l of SMART MMLV Reverse Transcriptase, and the mixture was incubated at 42 °C for 10 minuytes. Then 1 μ l of SMART III-modified oligo was added to the tube, and it was incubated at 42 °C for 1 hour. After last step, the first-strand synthesis was terminated by keeping at 75 °C for 10 minutes. When sample was cooled to room temperature, 1 μ l of RNase H (2 units) was added to the tube.

Before proceeding to the next step for LD-PCR amplification, the reaction should be incubated at 37 °C for 20 minutes. The PCR reaction should set up the PCR mix which were 2 µl of First-Strand cDNA, 70 µl of deionized water, 10 µl of 10X Advantage[®] 2 PCR Buffer, 2 µl of 50X dNTP mix, 2 µl of 5' PCR Primer, 2 µl of PCR Primer, 10 µl of 10X Melting Solution, and 2 µl of 50X Advantage 2 Polymerase Mix. The PCR reaction included 95 °C for 30 seconds, 20 cycle reaction, and 68 °C for 5 minutes. The cycle contained 95 °C for 10 seconds and 68 °C for 6 minutes, but the 6 minutes would increase 5 seconds after one cycle. Then the 93 µl of PCR product was added to the center of the flat surface of the gel matrix of one CHROMA SPIN TE-400 column which had already done the pre-treatment, and the column was centrifuged at 700 g for 5 minutes. The purified sample was transferred from collection of the spin column to a microcentrifuge tube, and the one tenth volume of sample of 3 M sodium acetate and 2.5 volume of sample of ice-cold 100% ethanol were added in the tube. After the mixture was kept at -20 °C for 1 hour, the sample was centrifuged at 14,000 rpm for 20 minutes at room temperature. When the supernatant was removed completely, the pellet dried by air was resuspended in 20 µl of deionized water. The cDNA was ready for library construction. (Clontech).

Yeast two hybridization

First of all, the Y2Gold strain competent cells (Clontech) were restreaked on YPDA plate (yeast peptone dextrose adenine plate) and incubated on 30 °C for three days, and the colonies appeared after three-day incubation. Single colony was picked and inoculated to 3 ml YPDA medium with 250 rpm shaking at 30 °C for 8 hours. Then the 5 µl of the yeast solution was transferred to 50 ml of fresh YPDA, and it was incubated at the same condition until the OD₆₀₀ value reaches 0.15-0.3. After the yeast

cells were centrifuged at 700 g for 5 minutes at room temperature, the pellet was resuspended in new YPDA and incubated at 30 °C with shaking until the OD₆₀₀ value reaches 0.4-0.5. The yeast cells were centrifuged at 700 g for 5 minutes at room temperature, and the pellet was resuspended in 30 ml of sterile water. Then the cells were centrifuged again at the same condition, and the pellet was resuspended in 1.5 ml of 1.1X TE/LiAc. Additionally, the cells were centrifuge at high speed for 15 seconds, and the pellet was resuspended in 600 µl of 1.1X TE/LiAc. The yeast cells were ready for co-transformation using the rainbow trout cDNA library with pGBKT7 and pGADT7 vectors.

The transformation mix, which contained 20 µl of library cDNA, 4 µl of pGADT7, and 2 µl of pGBKT7 harboring the *Zar1l* gene, transfer to a pre-chilled tube, and 20 µl of Yeastmaker Carrier DNA denatured by heating at 95 °C, 600 µl of yeast competent cells, and 2.5 ml of PEG/LiAc were added. Then the tube was incubated at 30 °C for 45 minutes, and the sample should be vortexed per 15 minutes. After 160 µl of DMSO was added to the sample, the tube was incubated in a 42 °C water bath for 20 minute, and the mixture was vortexed per 10 minutes. The cells were centrifuged at 700 g for 5 minute at room temperature, and then the pellet was resuspended in 3 ml of YPD Plus Medium. After the tube was incubated at 30 °C with shaking for 90 minute, the sample was centrifuged at 700 g for 5 minute at room temperature. The pellet was resuspended in 0.9%(w/v) NaCl Solution, and the yeast cells were cultured on selecting plates (Clontech).

The hybridized yeast cells were cultured on SD/-Trp, SD/-Leu, SD/-Trp/-Leu, SD/-His/-Trp/-Leu, and SD/-Ade/-His/-Trp/-Leu plates which contain X- α -Gal and Aureobasidin A, and they were incubated at 30 °C for three days. The blue colonies were picked and spread a square on a high dropout plate at 30 °C.

Yeast plasmid isolation

Yeast plasmid was isolated according the instruction of yeast plasmid isolation kit (Clontech). Half-cells of the colony were scooped and transferred to 200 µl of the supplied 10 mM EDTA, and the tube was centrifuged at 11,000 g for 1 minute. Then the pellet was resuspended in 100 µl of ZYM Buffer, and the 10 µl of Zymolyase suspension was added to the tube. After the sample was incubated at 30°C for 1 hour, the tube was centrifuged at 2000 g for 10 minutes. The pellet was resuspended in 125 µl of Y1 Buffer/RNase A solution, and then 125 µl of Y2 Lysis Buffer and 150 µl of Y3 Neutalization Buffer were added and mixed well in the tube. The sample was centrifuged at 11,000 g for 5 minute at room temperature, and the supernatant was transferred to Yeast Plasmid Spin Column. After the column was centrifuged at 11,000 g for 1 minute, the 450 µl of Y4 Wash Buffer was added to the column. When the flow-through was collected by centrifugation at 11,000 g for 3 minute, the 30 µl of YE Elution Buffer was added to the column. At the end, the yeast plasmid was collected by centrifugation at 11,000 g for 1 minute following incubation at room temperature for 1 minute.

Quantitative real-time polymerase chain reaction

The cDNA samples of ovary stage, embryonic development, and eggs of different quality were from the reverse transcription of the RNA isolation products treated with DNase. The real time PCR primers for *Zar1*, *Zar1l*, and endogenous gene (Table 1) were designed based on the corresponding cDNA sequences. Quantitative PCR was performed using the Bio Rad CFX96™ Real-Time PCR Detection System and the iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) in a 20 µl of reaction

volume containing 2 µl of cDNA sample, 5 µl of water, 3 µl of primer mix, and 10 µl of SYBR mix. The standard curves of *Zar1*, *Zar1l*, and endogenous genes were the 10-fold serial dilution of pooled cDNA samples. Each standard curve and cDNA sample have four repeats in one reaction. After the 2-step real time PCR, the Ct value, quantitative value, and melting curve were analyzed.

Statistical analysis

Standard curves for *Zar1*, *Zar1l*, and the endogenous control were constructed using 10 fold serial dilutions of PCR product. For each sample, the quantity of *Zar1*, *Zar1l*, and the reference gene was determined from respective standard curves. The quantity of *Zar1* and *Zar1l* was divided by the quantity of the reference gene to obtain a normalized value. The quantity of *Zar1* and *Zar1l* genes for vitellogenesis, embryonic development, and egg quality analysis was normalized by *hisH2A*, *GAPDH*, and β -actin, respectively. Mean differences in expression levels were reported as relative fold changes using the lowest expression value as calibrator. One-way ANOVA was performed to analyze *Zar1* and *Zar1l* expression using R software system. Multiple comparisons were used to determine the differences in *Zar1* and *Zar1l* gene expression among different stages. Different letters were labeled and indicated significant difference (P-value, 0.05).

Results

Sequence analysis of *Zar1* and *Zar1l* genes

The cDNA sequences of rainbow trout *Zar1* and *Zar1l* genes share some similarity (Fig. 2). The *Zar1* cDNA encodes a protein of 333 amino acids, and the *Zar1l* cDNA codes for a protein of 323 amino acids (Fig. 3). The amino acid sequence of *Zar1* and *Zar1l* proteins were analyzed using ClustalW. The sequence similarity of the two proteins is high at the C-terminal region of the sequences (Fig. 4). The sequence identities of rainbow trout *Zar1* protein with *Zar1* of several other species are over 40 %, and the identity of *Zar1* proteins between rainbow trout and zebrafish reaches 64 %. The identities of *Zar1l* protein of rainbow trout are also around 40 % with several species, and the identity of *Zar1l* protein of zebrafish is the highest with rainbow trout but only 59 % (Fig. 5). Moreover, both of *Zar1* and *Zar1l* protein of rainbow trout possess highly shared identity in C-termini with other species (data not show). The *Zar1* and *Zar1l* proteins in different species were analyzed by MEGA 5 software to construct a phylogenetic tree (Fig. 6). According to the phylogenetic tree, both proteins of rainbow trout have close relationship with zebrafish proteins. In the result, the species were separated to three main groups for both proteins.

Tissue distribution of *Zar1* and *Zar1l* genes

The distribution of *Zar1* and *Zar1l* genes in several tissues of rainbow trout was analyzed by RT-PCR. Expression of β -actin genes is similar in each tissue (Fig. 7), indicating the tissue samples are of good quality. Expression of *Zar1* gene is detected in multiple tissues including gill, liver, stomach, kidney, ovary, testis, skin, and heart, and significantly high expression of *Zar1* gene is seen in ovary and testis. In contrast, *Zar1l* gene is only expressed strongly in ovary tissue (Fig. 7).

Identification of proteins interacting with Zar1l protein

The coding region of *Zar1l* gene was cloned in frame with the Gal4 DNA binding domain to form a bait protein, and the libraries of prey proteins expressed the Gal4 activation domain. When the *Zar1l* protein interacts with the prey proteins, the transcription of four independent reporter genes is activated so that selection will take place on high stringency plate (SD/-ade/-his/-leu/-trp). There were five colonies that grew on the selecting plates, and they were cultured on high stringency plates with positive and negative controls (Fig. 8). The five proteins were sequenced and their identities were revealed. They are Di-N-acetylchitobiase, Serine/threonine/tyrosine-interacting protein (STYX), Ariadne-2 homolog, GH20 HexA HexB-like, and C-type mannose-binding lectin.

Quantitative analysis of *Zar1* and *Zar1l* gene expression

During vitellogenesis, the *Zar1* gene expression is high in pre-vitellogenesis stage, and low in early vitellogenesis stage. After early vitellogenesis, the *Zar1* gene expression starts to increase until the late vitellogenesis. *Zar1l* gene expression is also significantly high in pre-vitellogenesis, but it begins to decrease in the subsequent stages (Fig. 9). Real-time PCR analysis of *Zar1l* gene expression during embryonic development shows that *Zar1* gene expression is high in 0 h and starts to reduce until 10.5 h, but there is a significant peak in 19.5 h. After 19.5 h, the *Zar1* gene expression decreases again, and the expression is very low in 7d embryos. *Zar1l* gene expression is also high in 0 h and keeps reducing in the following stages (Fig. 10).

Quantitative analysis of *Zar1* and *Zar1l* gene expression in egg quality

The quantitative analysis of *Zar1* gene expression in eggs of various quality shows that *Zar1* gene expression is high in eggs at day1 post-ovulation (D1PO) and low in eggs at day 14 post-ovulation (D14PO). Similarly, *Zar1l* gene expression also is high in D1PO eggs but reduced in D14PO eggs (Fig. 11). The results indicate that *Zar1* and *Zar1l* gene expression is down regulated in eggs of reduced quality. Although the changes are not statistically significant, both genes have a reduction trend as the egg quality declines.

Discussion

Zar1 gene has been studied as a maternal-effect gene in several species, and these studies indicated that *Zar1* gene play a key role in early embryonic development. Additionally, it was also used to observe other effects of physiology, such as fertility and sex maturation (Wu, Viveiros et al. 2003; Michailidis, Argiriou et al. 2010). In this study, we identified and characterized *Zar1* gene in rainbow trout, and the *Zar1* gene was determined by real-time PCR for the expression levels in vitellogenesis, embryogenesis, and egg quality. *Zar1l* gene was identified in 2008 (Sangiorgio, Strumbo et al. 2008), but there are few studies that focused on it. According to my knowledge, our study is the first to characterize *Zar1l* gene in rainbow trout. In addition, we not only measured the expression levels of *Zar1l* gene during vitellogenesis and embryogenesis, and in eggs of different quality, but also found out the proteins that interact with *Zar1l* protein.

The structure of *Zar1l* protein is highly related to *Zar1* protein, and the C-terminal region possesses high similarity between not only these two proteins but also different species (Sangiorgio, Strumbo et al. 2008; Hu, Wang et al. 2010; Michailidis, Argiriou et al. 2010). Our study also shows that the C-terminal region is very similar between two proteins of rainbow trout, and both proteins have a conserved zinc-binding domain and an atypical PHD motif (C-X₂-C-X₁₃-C-X₂-C-X₄-C-X₁-C-X₁₇-C-X₂-C) (Fig. 4). PHD domains appear in two major classes of proteins: (i) transcriptional activators, repressors or cofactors and (ii) subunits of complexes that modulate chromatin (Wu, Viveiros et al. 2003). Thus we can assume that the *Zar1* and *Zar1l* proteins in rainbow trout may be a transcriptional regulator. The DNA sequences of two genes are also very close, especially in the open reading frame region. Furthermore, protein sequences of *Zar1* and *Zar1l* of rainbow trout were also

compared with other species, and the identity of whole proteins between different species was detected to be around 40%. If we only analyze the C-terminal region of the two proteins, the identity is very high. Moreover, the amino acid sequences of *Zar1* and *Zar1l* proteins from different species were analyzed to form phylogenetic tree, and these species were separated to three main groups. Rainbow trout is most closely related to zebrafish.

The distribution of *Zar1* gene was detected in several specie tissues, and the result shows that *Zar1* gene is expressed in multiple tissues (Wu, Viveiros et al. 2003; Wu, Wang et al. 2003; Brevini, Cillo et al. 2004; Pennetier, Uzbekova et al. 2004; Uzbekova, Roy-Sabau et al. 2006; Sangiorgio, Strumbo et al. 2008; Michailidis, Kalivas et al. 2009; Michailidis, Argiriou et al. 2010). In our study, *Zar1* gene is expressed in gill, liver, stomach, kidney, ovary, testis, skin, and heart tissue of rainbow trout, and the ovary and testis showed the highest level of expression. Thus the expression of *Zar1* gene in rainbow trout is like in other species primarily in gonad tissues. Additionally, *Zar1l* gene was only detected in ovary tissue in rainbow trout, which is different from *Zar1l* gene in cattle which was detected in multiple tissues (Sangiorgio, Strumbo et al. 2008). *Zar1l* gene is expressed in gonad tissues in chicken, but mouse also only can be detected in ovary tissue (Hu, Wang et al. 2010; Michailidis, Argiriou et al. 2010). Therefore the *Zar1* and *Zar1l* genes are mainly expressed in gonad tissues in every species.

Zar1 and *Zar1l* genes exhibit different expression patterns during vitellogenesis. Both genes are expressed in pre-vitellogenesis stage, but *Zar1* gene significantly drops down to low level in early vitellogenesis. The expression level of *Zar1* gene rises again in late vitellogenesis, while *Zar1l* gene decreases from early vitellogenesis to late vitellogenesis. Both genes were degraded after pre-vitellogenesis,

but *Zar1* is produced in late-vitellogenesis. The expression patterns of *Zar1* and *Zar11* genes in our study indicate that both genes possess high expression level in the beginning of embryonic development (0 h). Furthermore, *Zar1* and *Zar11* genes start to decrease in 7.5h, but *Zar1* gene shows a peak at 19.5h. This suggests that *Zar1* synthesis occurs at this stage. There is an increase in 4-cell stage for *Zar1* mRNA in cattle, and it was evidenced by semi-quantitative analysis (Brevini, Cillo et al. 2004). *Zar1* synthesis appears in distinct stage that may depend on different species. *Zar11* gene of rainbow trout continuously declines after 0h, and there is no peak during embryonic development. Other studies use RT-PCR to determine the expression of *Zar11* gene, and the bovine *Zar11* gene was not observed in embryonic development (Sangiorgio, Strumbo et al. 2008). The mouse *Zar11* gene was only found in zygote stage, but chicken *Zar11* gene is expressed during the entire embryonic development (Hu, Wang et al. 2010; Michailidis, Argiriou et al. 2010). According to these studies and our results, *Zar11* gene plays an important role in embryonic development for oviparous animals.

The mRNA expression of 39 genes was determined in post-ovulatory aged eggs, and the result exhibited different abundance of mRNA in deferent collection time (Aegerter, Jalabert et al. 2005). These expression differences of mRNA were induced by the post-ovulatory ageing, so I also wanted to detect the variation of *Zar1* and *Zar11* expression during post- ovulatory ageing. We used three groups for three time points of post-ovulation, and three groups also represent to three kinds of egg quality. The egg quality was defined as its ability to be fertilized, and subsequently develop into a normal embryo (Bobe and Labbé 2010), so the eggs are low quality for long time after post-ovulation in rainbow trout. The expression levels of *Zar1* and *Zar11* decrease with age post-ovulation or with declining fertilization ability of the

eggs. However, the decrease trend of *Zar1* and *Zar1l* gene expression during post-ovulatory ageing is not statistically significant, both genes cannot be an appropriate detector of egg quality. If among of replications in each egg qualities are increased, the result maybe will appear statistically significant change.

The Yeast-two hybridization assay shows five proteins interactive with *Zar1l* protein, and they are Di-N-acetylchitobiase, Serine/threonine/tyrosine-interacting protein (STYX), Ariadne-2 homolog, GH20 HexA HexB-like, and C-type mannose-binding lectin. The Di-N-acetylchitobiase was associated with the lysosomal fraction of the liver homogenate, and it cold cleavage triantennary chains and di-N-acetylchitobiose (Kuranda and Aronson 1986). Additionally, Di-N-acetylchitobiase is involved in the degradation of asparagine-linked glycoproteins. STYX is a unique modular domain found within proteins implicated in mediating the effects of tyrosine phosphorylation in vivo (Wishart and Dixon 1998), and the archetype STYX/dead-phosphatase that is necessary for normal sperm produce will complex with a spermatid mRNA-binding protein (Wishart and Dixon 2002). In addition, Ariadne-2 homolog (ARIH2) conserved an IBR (In Between Ring fingers) domain that often occurs between two pairs of ring fingers. The protein containing two ring fingers and one IBR domain is termed RBR family protein, and the RBR family members are involved in the protein quality control and indirectly regulate transcription. Furthermore, GH20 HexA HexB-like protein was analyzed by CD search. N-acetyl-beta-D-hexosaminidase A (HexA) and beta-N-acetylhexosaminidase B (HexB) were encoded from the hexA and hexB genes, and the both subunits have a TIM-barrel fold and belong to the glycosyl hydrolase family 20 (GH20) (NCBI). Moreover, C-type mannose-binding lectin (MBL) is an important component of innate immunity in mammals, and it was produced by liver hepatocytes. When there are an infection or

inflammatory response, MLB will increase the production. MBL is involved to the phagocytosis by macrophages and also activates the mannan-binding lectin complement pathway of the innate immune response (Ourth, Narra et al. 2007). Especially, STYX expression has negative regulation on growth factor mediated activation of specific MAPKs (mitogen-activated protein kinase) in mammalian cell culture (David Karnak 2008). MAPKs may be an important regulator in MI-MII transition, pronucleus formation and the initiation of the first mitosis in pig eggs (Manyu Li 2002). *Zar1l* also can be detected in GV oocytes to 4-cell embryo in mouse (Hu, Wang et al. 2010), therefore, by interacting with STYX protein, *Zar1l* might involved in the oocyte development of rainbow trout. Moreover, *Zar1l* protein conserves a zinc-binding domain and an atypical PHD motif, and it can interact with these proteins to create a function or effect.

In conclusion, *Zar1* and *Zar1l* of rainbow trout are structurally related and share some sequence identity. Both genes are primarily expressed in ovary tissue, and the expression levels decrease as the egg quality declines. Additionally, *Zar1* synthesis occurs in 19.5h, but *Zar1l* degrades during embryonic development. The expression of *Zar1* gene declines after pre-vitellogenesis and increase again in late-vitellogenesis, while *Zar1l* gene declines throughout vitellogenesis. Moreover, the result of yeast-two hybridization system indicates *Zar1l* protein has interaction with Di-N-acetylchitobiase, Serine/threonine/tyrosine-interacting protein (STYX), Ariadne-2 homolog, GH20 HexA HexB-like, and C-type mannose-binding lectin. Further studies should focus on the effect and function of the interaction between *Zar1l* protein and the five proteins.

Tables and Figures

Table 1. Primers used in this project

PCR method	Primer name	Primer sequences	Product Size
Regular PCR	RT-AZar1 F453	GCTGTCTACTCGCCTGTAGCTT	259 bp
	RT-AZar1 R712	GTCTTGACTTCCGATCCATCC	
	RT-OZar1 F580	GAAAACGGAGGACCAGGTTAG	425 bp
	RT-OZar1 R1005	AGGTCTGTAGCAGGGATGGTT	
	TroutActin F911	AAGTGTGACGTGGACATCCGT	510 bp
	TroutActin R1421	TAATCCGCTGCTTCACCGTTC	
Real Time PCR	rtZar1 F915	TGCGTGTGCGAAGTGACCCC	169 bp (For embryo development)
	rtZar1 R1084	TGGGCTGCACTTAGATGTGACA	234 bp (For ovary stage and eqq quality)
	rtZar1 F453	GCTGTCTACTCGCCTGTAGCTT	
	rtZar1 R687	CTTTGACATCCTCGGTTCGTAA	195 bp
	rtZar1like F241	CGGGGTTTCAGGTCAACGCCC	
	rtZar1like R436	TCGCGAGAAGCGCACGTTGT	72 bp (For egg quality)
	TroutActin rtF541	GCCGGCCGCGACCTCACAGACTAC	
	TroutActin rtR613	CGGCCGTCCTCCTGAAGCTGTAAC	343 bp (For ovary stage)
	hisH2a F212	CCCGTGACAACAAGAAGACTC	
	hisH2a R555	CCTCATGTGGGCACTTGATAC	For embryonic development
	GAPDH Forward	CCCTCAAGGTTGTCAGGAAT	
	GAPDH Reverse	CCTCCACAGCTTTCAGAAAG	971 bp
Yeast Two Hybridization	Zar1L-NdeI	CCCCATATGATGTTTTCTCCATACAACTTTAAT	
	Zar1L-BamHI	CCCGGATCCTCAGACAATGTATTTGTAGCTATA	

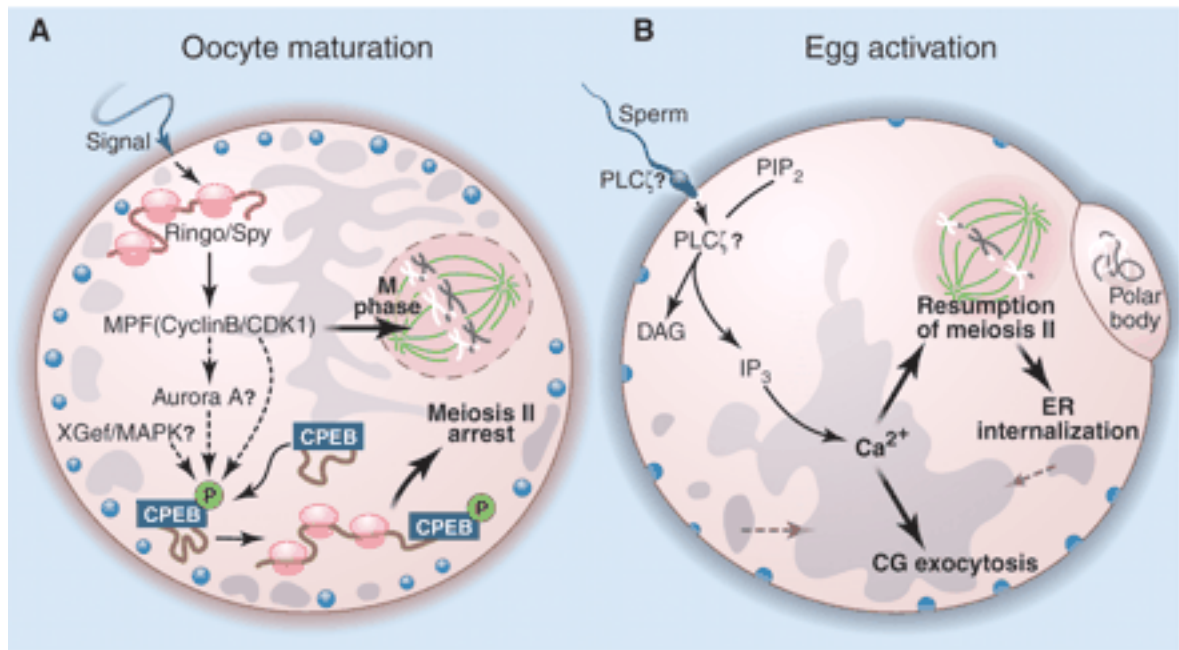


Figure 1. The regulation mechanism of oocyte maturation and egg activation in vertebrates. A) An extracellular factor causes oocyte maturation and stimulates the translation of specific mRNA. B) The fertilization triggers egg activation in the matured oocyte (Stitzel and Seydoux 2007).

TC132527: Zar1
TC133893: Zar1 like

```

TC132527      AATCGGTAAACCAATAACTCAAGTTGGCTGAGAAAAATGGCTACATATGTACACGAGT 60
TC133893      ----GGGGTTTATGTTTCTCCATACAAC-----TTAATGGCTATCCGGGCTGCG---- 47
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      CGATCCACAGTTATTTCTATTCTATT-ACAACCCCTTATCTGGCAGGTATCCCAAACCA 119
TC133893      -GTACCATGATGGCTCCTATCTCCACGGCAACCCGAAATT--CAAG--CCTCACACCT 101
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      AAAGTGCGGGCTGGAGAAACAATTATTTGTCAACTATGTGGTACAGAGGACTATTTT 179
TC133893      G-----GGGTAAGGGGAGCATCTACGTGCTCCCGAAG-----CGCTGGACTACCTT 148
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      GATAACCACCAAGCGCGGCAACTTAAATCCATCCTGTCTCAAAATAACCCCAATCTTACA 239
TC133893      GACGTATGTAAACGGGCCAGCTGAAGGCCATCCTGTCTCAGGTGAATCCCAACCTTACC 208
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      CCGAGGCTACGGAAAGCAAAATACCAAGATGTCTGCTGTGCAGGTCAACCCGAAAGAGAT 299
TC133893      CCTCGCTCTCGGAAGGCAAAACCAAGAGGTTCGGGGTTCAGGTCAACGCCAGGTGCAC 268
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      CTCTCCGTCTCAGTGCTCCCTCGGCCAAGGACTCTCATG-ACCGGAAGCGAGACGCTTT 358
TC133893      GCGATGGTCCAGTGCTCCCTCGGACCCAGAGCGCTGTTCTACCGGGAGAGGAAATTTCCG 328
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      GCGCAAGAGGAAACAGGAGGCCCGAGCCACCGGTAGCCCTGGGGGTAGTCCAGGCGAGCT 418
TC133893      GTATTTTTCGAGTGCCTGTGAAGTGCCAGCAGAGTCCCTCCGCGCTTCTTCTCTGGAC 388
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      GGTGGGAGCGGTGCTCTACCTCGCAGACTGGCTGTCTACTCGCTGTAGCTTCTAGGAG 478
TC133893      --CGAAGACGT-----TGCCGAGCACTCCGGTAAACACGTGG-----CTTCTCG-CG 435
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      ACTTGCAATATTTCTAGAAGACGACAAAGGGGACCCGAGTGAAGCACAAACCGGTGACCC 538
TC133893      ACCC--CTGCCATCTACTCTCCGGTGTTCATCGCCGTTCTTCGCGCTGCCGGTGAACG 494
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      ACCTG--AAACCGTGAAGGCCCGGAAAGAAATAAAGCGCAAAAGTCTGCGGAGGACAGA 596
TC133893      CGCAGGATCAAGCGTGTGCTGTGAGGAGAAAGTGC-----CGCAACG-GGGCTAGT 547
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      ACAGAAAGCGCAAAGGACGAAAGACTGGGCCAGGTAAAGAACTAGTAAAGACTGAC 656
TC133893      GATGAGGATGCGCAGGCCGACGTTACCGAGCAG-----AAAACGGAGACCGGTTAGAC 602
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      CGGTCAAGTTACGACCGAGGATGTCAAAGCCGATCAGGATGGATCGGAAGTCAAGACCGG 716
TC133893      CGGATA--TCCGCGCTGAGGTTGTGAGGAAACCCCTACACAGCCGCGCAAGGTTTAAAC 661
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      GTGAGGTTTTCAGTTTCTGGAGCAGAAGTATGGGTACTTACTGCAGAGACTGCAACCTG 776
TC133893      -----TTTCAGTTTCTGGAGCAGAAGTACGGAATTTTCCACTGCAGCCAGTGAAGCT 715
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      CGTTGGGAGAGCGCATATGTTTGGTGCCTCAAGGAACGAGCAAGGTCTACTTCAAGCAG 836
TC133893      CGTTGGGAGAGTGCCTATGTGTGGTGCATCTCTGGAACAGTAAAGTCTACTTCAAGCAG 775
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      TTTCTGTAGAACGTGCCAGAAGTCTTCAATCCTTACCGGGTGCAGGACATCACTGCGAG 896
TC133893      CTCTGTCGTAAAGTCCAGAATGGATTTAAACCCCTACAGAGTGGAGTCTATCCAGTGAAG 835
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      ACTTGCACAAAGTACCGCTGCGTGTGCGAAGTGACCCCTCGCCACATTGACCCCAACGC 956
TC133893      GTGCTTCCAGACCCGGTGTGCTGTGAGCAGAGGAGAGCACATTGACATGAAGAGA 895
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      CCCACAGACAGGATCTGTGTGGCAGGTGAAGGGCAAGCGGCTCTCCTGTGACCGCACCC 1016
TC133893      CCTCATAGACAGGACCTGTGTGGCCCTGCCGGGGAAGAGGATTTCTGTGACACTACC 955
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      TTTCAATTTCAAGTACATCATCT-AGCAGGAACCTGCACATGAAGTGTACATCTAAG-- 1072
TC133893      TATAGCTACATATACATTTCTGTGAATTAACCATTCCTGCTACAGACCTAATAACAGAT 1015
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      -----TCTTCGACCC----- 1082
TC133893      CTAGGATCAGATTGAGGCCCATCACCACCAACATCCATACTTTTGGTTGACATATCTGA 1075
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      -----CAGAC-----AAGCCCCG-----TGCACATT 1104
TC133893      ATTATTAGATCAGTGTAGTGGAAGATGGATGTAAACCAAGTTTCTAATCCTGGACTTA 1135
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      TAAAGAGG-----CTCTGGGTGGAGGAGAACTACTT----- 1135
TC133893      AAAGGGGTGCATGTTTTTCCCTTTGCTTTAACACCTGACTCGATGAATATACTTGAGTT 1195
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      -TTGGATTTTCATT----- 1147
TC133893      GATGAATTTAATTTGGATGTTAGAGCTAGGGCAACCAATACATGTGCACCCCTTTGGGTG 1255
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      ----- 1181
TC133893      CCAGGATGTGACGATTGCCGTAAGCACAGACTAGGATCTGATTAGGGAAGTGCCAA 1315
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      -----ATTTCCTG-----TTATGCCCTTTATTT----- 1170
TC133893      TCCTAAACCTTACTGCCCTTGGTGGGATGCACAACCTGACTTTATACAGTATCTAGAGCC 1375
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      -----CATTATGATAG----- 1181
TC133893      ACAGTATCCTACTCTGAGCCTTCACATGACAGGCTAAACAACACTATGCCACAGAGGT 1435
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      -----CAGGAAACACTTCT----- 1196
TC133893      AGTTGACCTTAACCACATATCTAGGATCAGGTCAACCTTCTCCGATCCTATCCACGGTT 1495
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      GAATGTGAATAAAGTGG-----CAATACTCAGC----- 1225
TC133893      GTTAGGAGGTGCAGTGGTCAACTGTTTCTTACCTGCCAAGATCTGTCCACGCTTAA 1555
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      --AGTCT----- 1230
TC133893      ATAGTCTGCCCTCATAACTTTTATACTCTTTCCACCGTGATCACTGAGTGACTCAACTTT 1615
      * . . . . . * . . . . . * . . . . . * . . . . .
TC132527      ----- 1622
TC133893      TTTTGTGA 1622

```

Figure 2. The comparison of the cDNA sequences of *Zar1* and *Zar1l* genes in rainbow trout. The sequences of *Zar1* and *Zar1l* genes were analyzed by BioEdit software.

TC132527: *Zar1*

```

39 atggctacatatgtagacgagtcgacagttatttctattca
M A T Y V D E S I D S Y F Y S
84 tcttacaacccttattctggcaggtatcccaacccaaagggtg
S Y N P Y S G R Y P K P K G A
129 ggctgggaaacaattattgtccaactatgtggtacagaggac
G W R N N Y L S N Y V A T E D
174 tttttgataaccaccagcgcgcaacttaaatccatcctgtct
Y F D N H Q R A Q L K S I L S
219 caaataaaccccaattttacacggaggtacggaagcaaatacc
Q I N P N L T P R L R K A N T
264 aaagatgtcgtgtgacaggtcaacccgaagaggtgtctccgtg
K D V A V Q V N P K K D V S V
309 cagtgtcctcccgcccaaggaactctcatgacggaaagcgagac
Q C S L G P R T L M T G K R D
354 gctttgcgaagaggaacacaggagggccaggaccccggtagccct
A L R K R K Q E A Q A F G S F
399 gcgggtagtcacggcagctcggtgggagcggtgctctacccctgc
A G S P G S S V G G V L Y P R
444 acactggctgtctactcgctgtagcttctaggagacttgcatat
T L A V Y S P V A S R R L A Y
489 tttctagaagacgacaaagggtgacggagtgaaagcacaacccgt
F L E D D K G G P S E A Q T G
534 gacccacctgaacccgtgaaggccgggaaaaagataaaggcgaa
D P P E T V K A G K K N K G E
579 aagtctgcggaggacagaacagaaaaggcaaggacgaaaagact
K S A E D R T E K A K D E K T
624 ggccacaggtaaaaaaactagtaagaagactgacccgtcagttacg
G P G K K T S K K T D R S V T
669 accgaggtatgtcaaacgcatcaggatggatcggaagtcagacc
T E D V K A D Q D G S E V K T
714 cgcgtgaggtttcagtttctggagcagaagtatgggtactatcac
R V R F Q F L E Q K Y G Y Y H
759 tgcagagactgcaacctgcgttgggagagcgcatatgtttgtgc
C R D C N L R W E S A Y V W C
804 gtccaaggaacagcaaggtctacttcaagcagttctgtagaacg
V Q G T S K V Y F K Q F C R T
849 tgccagaagtctctcaatccttaccgggtcgaggacatcacctgc
C Q K S F N P Y R V E D I T C
894 cagacttgcaacaagtcaagtcggtgtgcgaagtgacccctgcg
Q T C N K S R C V C E V T P R
939 cacattgaccccaaacgccccacagacaggtatcgtgtggcagg
H I D P K R P H R Q D L C G R
984 tgtaagggaagcggtctctctgtgacccgacccctcagtttcaag
C K G K R L S C D R T F S F K
1029 tacatcatctag 1040
Y I I *

```

TC133893: *Zar1* like

```

8 atgtttttccatacaactttaatggctatccgggctgcggtacc
M F S P Y N F N G Y P G C G T
53 atgatggctcctatcctccacggcaacccgaagttcaagcctcac
M M A P I L H G N P K F K P H
98 acctggggtaaggggagcatctacgtgcctcccgagcgctggac
T W G K G S I Y V P P E A L D
143 taccttgacgtatgtaaaacggggcccagctgaaggccatcctgtct
Y L D V C K R A Q L K A I L S
188 caggtgaatcccaaccttaccctctgtctccggaaggcaaacacc
Q V N P N L T P R L R K A N T
233 aaggagttcgggggttcagggtcaacgcccaggtcgacgcgatggtc
K E F G V Q V N A Q V D A M V
278 cagtgtcctcccgacccaagaacgctgttctacccgggagagaaa
Q C S L G P K T L F Y R E R K
323 tttccggtattttcgaagtcgcctgtggaagtgccagcagagtcctc
F P V F S K S P V K C Q Q S P
368 tcccggtcttctctctggacccgaagcggtgcccagcactccg
S A A S S L D P K A L P S T P
413 gtaaacacgtgcgcttctcgcgacccctcgccatctactctccg
V N N V R F S R P L A I Y S P
458 gtgtttgatcgccggttcttccgcgtgcgggtgaacgcgaggtat
V F D R R F F A L P V N A Q D
503 gacagcgtgtgtgtgagggagaaggtggcggaacggggcctagt
D S V C C E G E G G N G A S
548 gatgaggtatggcgagggccgacgttacgcagcagaaaaacggaggac
D E D G E A D V T E Q K T E D
593 caggttagacggatattcccgctgaggtgtcagggaaaccccta
Q V R P D I P R E V V R K P L
638 caccagccgccccaaagggttcaactttcagtttctggagcagaag
H Q P P K G F N F Q F L E Q K
683 tacggatttttccactgcagccagtgtaacgttcggttgggagagt
Y G F F H C S Q C N V R W E S
728 gcctatgtgtgtgcatctctggaactagtaaggtgtacttcaag
A Y V W C I S G T S K V Y F K
773 cagctctgtcgtgaagtgcagaatggatttaacccctacagagtgt
Q L C R K C Q N G F N P Y R V
818 gagtctatccagtgaaggtgtgttccagaccccggtgtgtgtgt
E S I Q C K V C S Q T R C C C
863 gagcagaagagagggcacattgacatgaagagacctcatagacag
E Q K E R H I D M K R P H R Q
908 gacctgtgtggcgctgcgggggaaaggaggttctctgtgacact
D L C G R C R G K R I S C D T
953 acctatagctacaaatacattgtctga 979
T Y S Y K Y I V *

```

Figure 3. *Zar1* and *Zar1l* protein sequences. The protein sequences were predicted based on the open reading frames of *Zar1* and *Zar1l* genes. The *Zar1* gene encodes a protein of 333 amino acids, and the *Zar1l* gene codes for a protein of 323 amino acids.

```

Zarl1      MATYVDESIDSYFYSSYNPYSGRYPKPKGAGWRNNYLSNYVATEDYFDNHQRAQLKSILS 60
Zarl1-like MFSPYNFNGYPGCGTMMAPILHGNPKFKPHTWGRKSIYVPPEALDYLDVCKRAQLKAILS 60
          * : : . . : * ** * * :. : : ** : *****:***

Zarl1      QINPNLTPLRLKANTKDVAQVNPVKDVSVCSLGPRTLMTGKR--DALRKRKQEAQAPG 118
Zarl1-like QVNPNTPLRLKANTKEFGVQVNAQVDAMVQCSLGPKTLFYRERKFPVFSKSPVKCQQSP 120
          *:*****:..****.: *. *****:*. :* .: * :.* .

Zarl1      SPAGS-----PGSSVGGVLYPRTLAVYSPVASRRLAYFLEDDKGGPSEAQTGDPPETVK 172
Zarl1-like SAASSLDPKALPSTFPVNNVRFSLAIYSPVFDRLFFALPVNAQDD-SVCCEGE-----G 174
          *.*. * :..*.* :.*.***:*** .*: : : :.. * . * :

Zarl1      AGKKNKGEKSAEDRTEKAKDEKTGPGKKTSKKTDERSVTTEDEVKADQDGSEVKTRVRFQFL 232
Zarl1-like GGNGASDEDEADVTEQKTEDQVRP-----DIPREVVRKPLHQPPKG-----FNFQFL 222
          .*: ..*.. * **: :.:. * . *.*. : :. :.* ..****

Zarl1      EQKYGYHCRDCNLRWESAYVWCVQGSTSKVYFKQFCRTCQKSFNPYRVEDITCQTCKNSR 292
Zarl1-like EQKYGFFHCSQCNRWESAYVWCISGTSKVYFKQLCRKCQNGFNPYRVESIQCKVCSQTR 282
          *****:.* :*:*****:..*****:*.**.*.*****.* *:.*.:*

Zarl1      CVCVTPRHIDPKRPHRQDLCGRCKGKRLSCDRTFSFKYII 333
Zarl1-like CCCEQKERHIDMKRPHRQDLCGRCRGKRISCDTTSYKYIV 323
          * ** . **** *****:***:*** *:***:

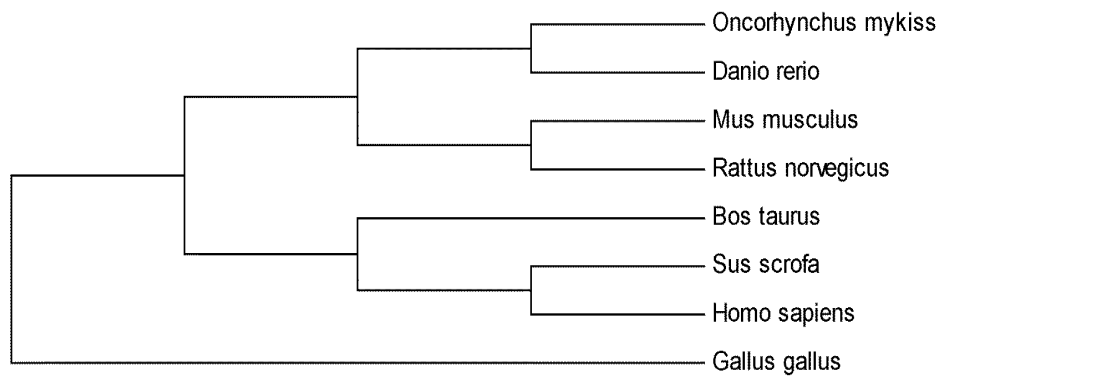
```

Figure 4. Comparison of amino acid sequences between *Zarl1* and *Zarl1l* proteins. The amino acid sequences of *Zarl1* and *Zarl1l* proteins are very similar at the C-terminal region. The asterisks indicate the same amino acids between two proteins. The alignment was created using ClustalW. The red color indicates not only the 12 conserved cystines but also an atypical PHD motif.

	Rainbow trout	Human	Mouse	Rat	Frog	Zebrafish	Pig	Cattle	Chicken	Zar1-like
Rainbow trout		45	39	40	45	59	43	42	47	Rainbow trout
Human	42		57	60	46	42	73	67	52	Human
Mouse	44	52		76	42	39	52	52	46	Mouse
Rat	45	55	88		38	38	55	55	48	Rat
Frog	57	46	45	48		45	41	42	49	Frog
Zebrafish	64	41	43	44	59		42	47	47	Zebrafish
Pig	46	65	53	54	51	46		69	50	Pig
Cattle	45	62	56	59	52	44	77		49	Cattle
Chicken	43	76	72	51	55	57	74	43		Chicken
Zar1	Rainbow trout	Human	Mouse	Rat	Frog	Zebrafish	Pig	Cattle	Chicken	

Figure 5. Amino acid sequence identity of *Zar1* and *Zar1l* proteins between different species. The left side indicates the percentage identity of amino acids in the entire *Zar1* proteins between different species, and the right side is for *Zar1l* protein. The identities of both *Zar1* and *Zar1l* proteins of rainbow trout are high with zebrafish.

A.



B.

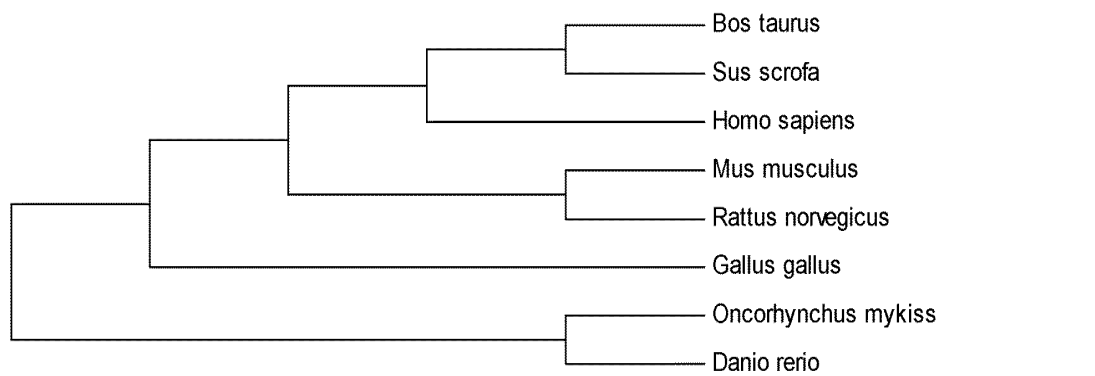


Figure 6. Neighbor joining phylogenetic trees of *Zar1* and *Zar1l* proteins. A) The phylogenetic tree of *Zar1* protein shows the relationship between ten species. B) The phylogenetic tree of *Zar1l* protein indicates the relationship between nine species. Rainbow trout possesses high connection with zebrafish.

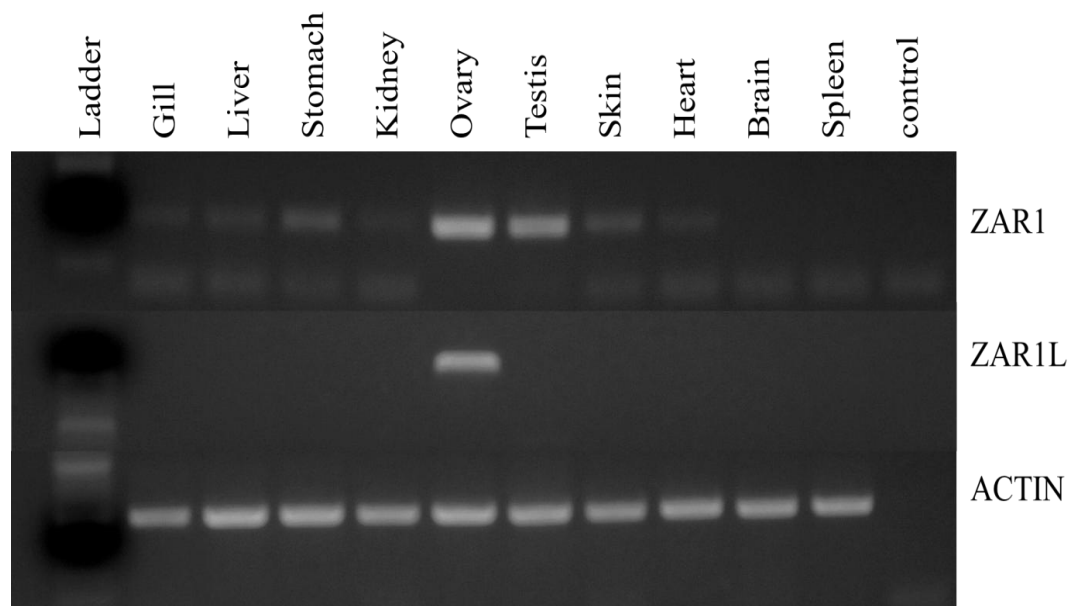


Figure 7. RT-PCR analysis of tissue distribution of *Zar1* and *Zar1l* genes. *Zar1* gene is expressed in grill, liver, stomach, kidney, ovary, testis, skin, and heart of rainbow trout, and the expression levels are higher in ovary and testis than other tissues. *Zar1l* gene is only expressed in ovary tissue of rainbow trout. β -actin was used as control for cDNA quality.

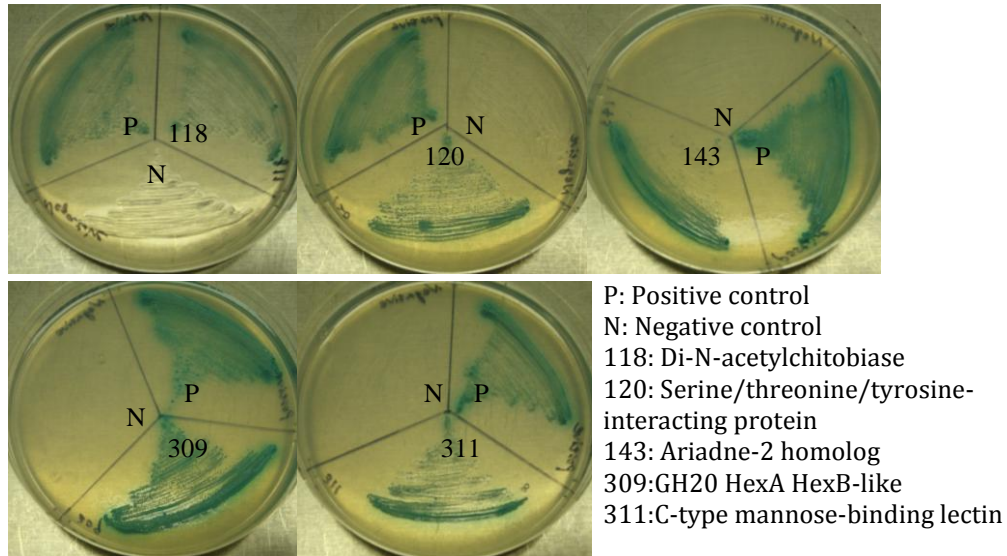


Figure 8. Yeast-two hybridization analysis for protein-protein interaction of *Zar1l*. Yeast-two hybridization assays identified five positive clones. Plasmids from these clones were isolated and used to transform yeasts that were cultured on high stringency plates with positive and negative control colonies. The five clones show green colonies on high stringency plates. The positive control has the same grow situation as the identified clones.

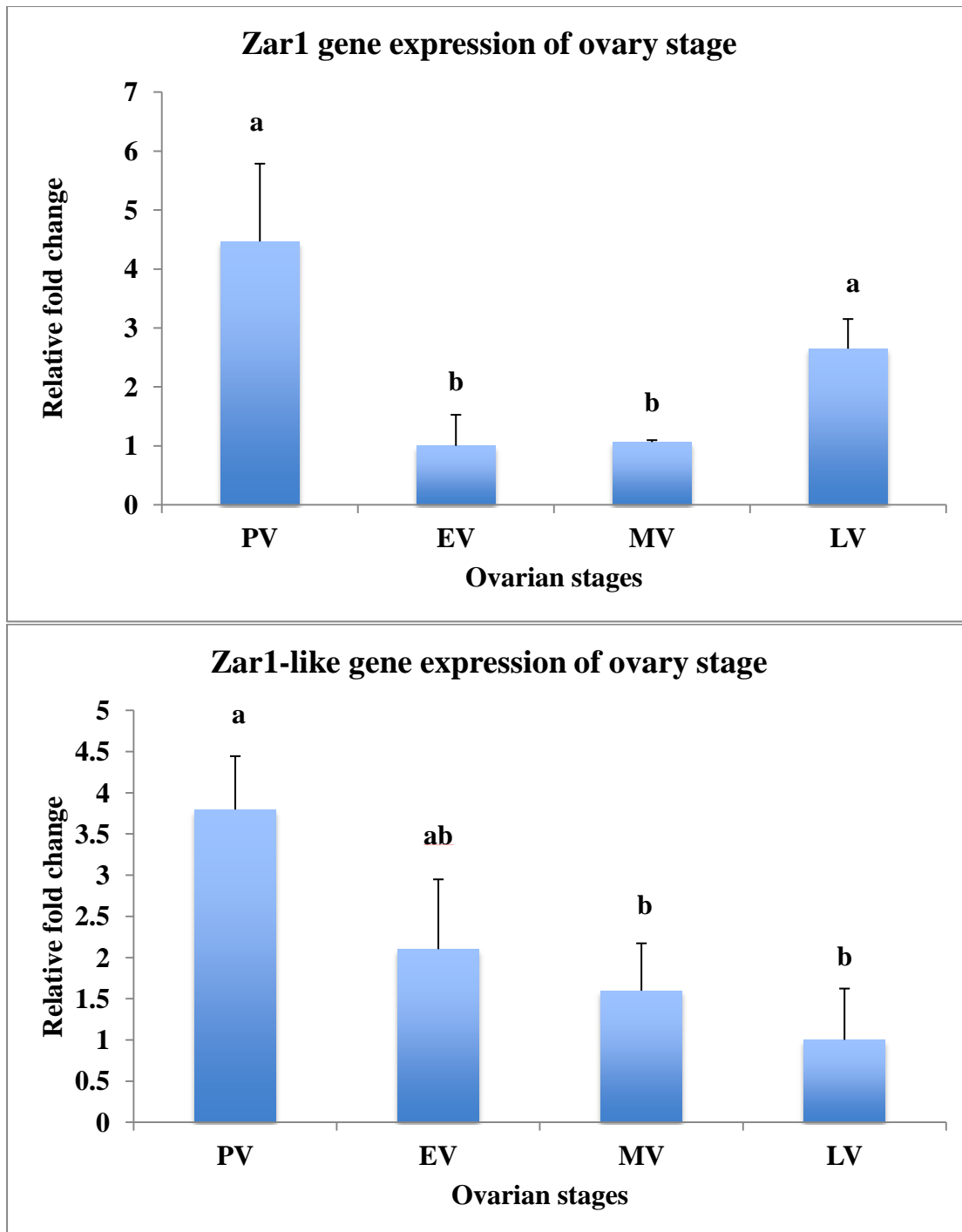


Figure 9. Real-time PCR analysis of expression levels of *Zar1* and *Zar1l* genes in ovarian stages. A) The expression level of *Zar1* gene in ovarian stages. B) The expression level of *Zar1l* gene in ovarian stages. The *Zar1* and *Zar1l* genes have high expression in pre-vitellogenesis stage (PV), and they both reduce in early-vitellogenesis (EV) and middle-vitellogenesis (MV) stages. However, the expression of *Zar1* gene will increase in late-vitellogenesis (LV). *Zar1l* gene still reduces in late-vitellogenesis.

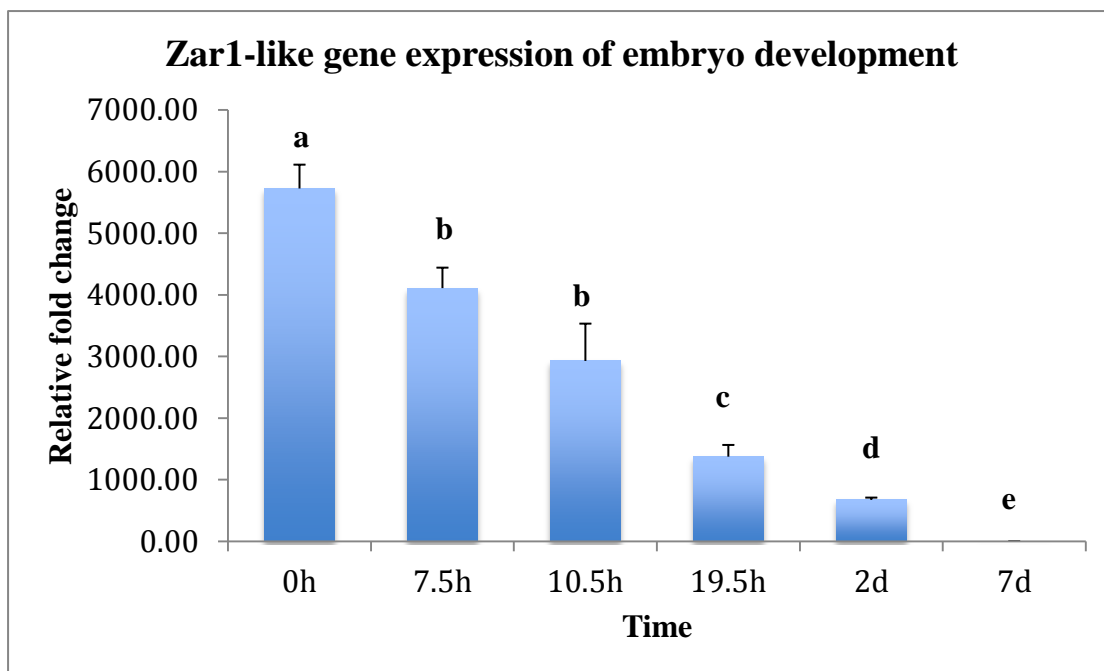
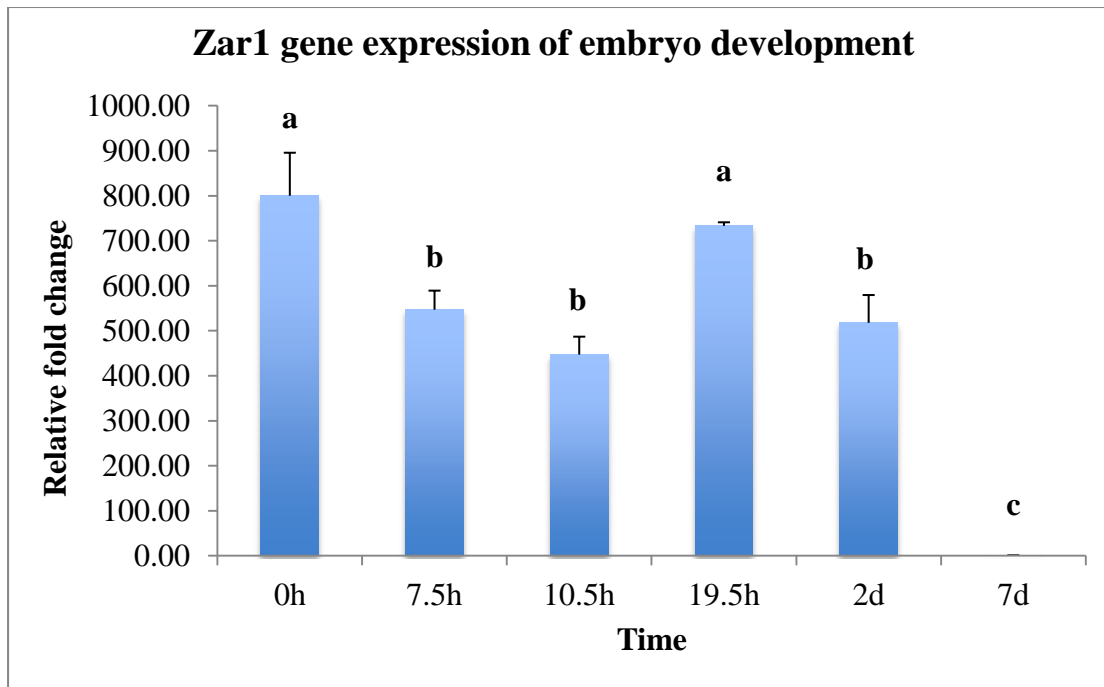


Figure 10. Real-time PCR analysis of expression levels of *Zar1* and *Zar1l* genes in embryonic development. A) The expression level of *Zar1* gene in embryonic development. B) The expression level of *Zar1l* gene in embryonic development. *Zar1* and *Zar1l* genes express highly in the beginning of embryonic development. The *Zar1l* gene continually reduce during the embryonic development, but the *Zar1* gene will have a peak in 19.5-hour.

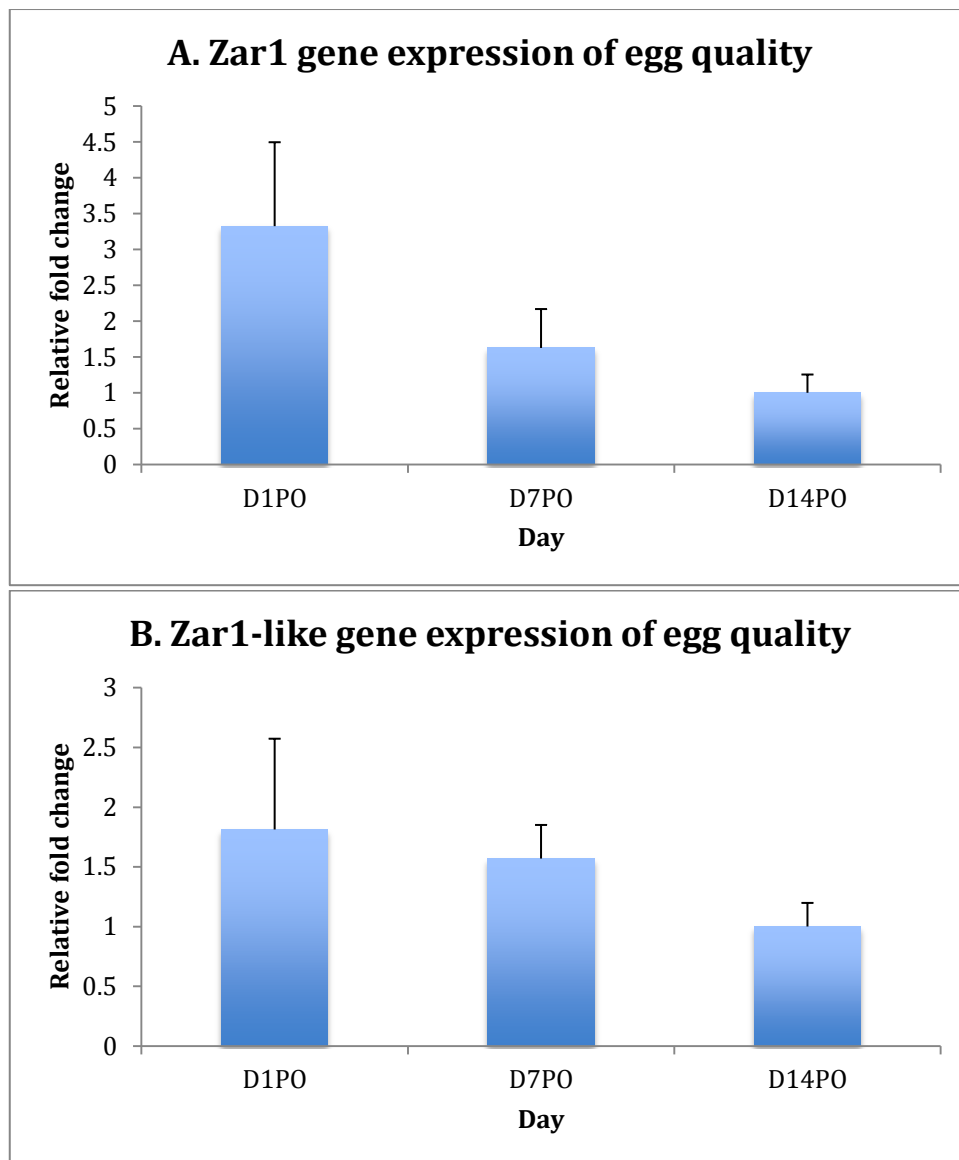


Figure 11. Real-time PCR analysis of *Zar1* and *Zar1l* gene expression in eggs of different quality. The expression levels of *Zar1* (A) and *Zar1l* (B) genes in eggs of different quality were analyzed by real time PCR. Both of the expression levels of *Zar1* and *Zar1l* genes are high in Day-1 post-ovulation, and the expression levels of *Zar1* and *Zar1l* genes reduce as the egg quality reduce.

References

- Aasland R., G. T. J., Stewart A.F. (1995). "The PHD finger: Implications for chromatin-mediated transcriptional regulation." Trends in Biochemical Sciences **20**(2): 56-59
- Aegerter, S. and B. Jalabert (2004). "Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss*." Aquaculture **231**(1): 59-71.
- Aegerter, S., B. Jalabert, et al. (2004). "Messenger RNA stockpile of cyclin B, insulin - like growth factor I, insulin - like growth factor II, insulin - like growth factor receptor Ib, and p53 in the rainbow trout oocyte in relation with developmental competence." Molecular reproduction and development **67**(2): 127-135.
- Aegerter, S., B. Jalabert, et al. (2005). "Large scale real - time PCR analysis of mRNA abundance in rainbow trout eggs in relationship with egg quality and post - ovulatory ageing." Molecular reproduction and development **72**(3): 377-385.
- Avery, T. and J. Brown (2005). "Investigating the relationship among abnormal patterns of cell cleavage, egg mortality and early larval condition in *Limanda ferruginea*." Journal of fish biology **67**(3): 890-896.
- Bally-Cuif, L., W. J. Schatz, et al. (1998). "Characterization of the zebrafish Orb/CPEB-related RNA-binding protein and localization of maternal components in the zebrafish oocyte." Mechanisms of development **77**(1): 31-47.

Bebbere, D., L. Bogliolo, et al. (2008). "Expression pattern of zygote arrest 1 (ZAR1), maternal antigen that embryo requires (MATER), growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) genes in ovine oocytes and in vitro-produced preimplantation embryos." Reproduction, Fertility and Development **20**(8): 908-915.

Blom, J. and K. Dabrowski (1995). "Reproductive success of female rainbow trout (*Oncorhynchus mykiss*) in response to graded dietary ascorbyl monophosphate levels." Biology of Reproduction **52**(5): 1073-1080.

Bobe, J. and C. Labbé (2010). "Egg and sperm quality in fish." General and Comparative Endocrinology **165**(3): 535-548.

Bonnet, E., A. Fostier, et al. (2007). "Characterization of rainbow trout egg quality: a case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations." Theriogenology **67**(4): 786-794.

Bonnet, E., A. Fostier, et al. (2007). "Microarray-based analysis of fish egg quality after natural or controlled ovulation." Bmc Genomics **8**(1): 55.

Brevini, T. A. L., F. Cillo, et al. (2004). "Expression pattern of the maternal factor zygote arrest 1 (Zar1) in bovine tissues, oocytes, and embryos." Molecular reproduction and development **69**(4): 375-380.

Bromage, N., J. Jones, et al. (1992). "Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout (*Oncorhynchus mykiss*)."
Aquaculture **100**(1-3): 141-166.

Brooks, S., C. R. Tyler, et al. (1997). "Egg quality in fish: what makes a good egg?"
Reviews in Fish Biology and Fisheries **7**(4): 387-416.

Burns, K. H., M. M. Viveiros, et al. (2003). "Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos." Science **300**(5619): 633-636.

Capili, A. D., D. C. Schultz, et al. (2001). "Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains." The EMBO journal **20**(1): 165-177.

Christians, E., A. Davis, et al. (2000). "Embryonic development: Maternal effect of Hsf1 on reproductive success." Nature **407**(6805): 693-694.

Ciereszko, A., M. Wojtczak, et al. (2009). "A lack of consistent relationship between distribution of lipid droplets and egg quality in hatchery-raised rainbow trout, *Oncorhynchus mykiss*." Aquaculture **289**(1-2): 150-153.

David Karnak, B. C., Knut Martin Torgersen and Matthew J Wishart (2008). "Roles for STYX in MAPK signaling and nuclear transport during germ cell maturation." The FASEB Journal **22**: 1048.1048.

Dean, J. (2002). "Oocyte-specific genes regulate follicle formation, fertility and early mouse development." Journal of reproductive immunology **53**(1): 171-180.

DeRenzo, C. and G. Seydoux (2004). "A clean start: degradation of maternal proteins at the oocyte-to-embryo transition." Trends in cell biology **14**(8): 420-426.

Dietrich, G. J., M. Wojtczak, et al. (2007). "Broken eggs decrease pH of rainbow trout (*Oncorhynchus mykiss*) ovarian fluid." Aquaculture **273**(4): 748-751.

Evsikov, A. V., J. H. Graber, et al. (2006). "Cracking the egg: molecular dynamics and evolutionary aspects of the transition from the fully grown oocyte to embryo." Genes & development **20**(19): 2713.

Fauvel, C., Omnes, M.-H., Suquet, M., Normant, Y. (1993). "Reliable assessment of overripening in turbot (*Scophthalmus maximus*) by a simple pH measurement." Aquaculture **117**: 107-113.

FitzHarris, G., P. Marangos, et al. (2003). "Cell cycle-dependent regulation of structure of endoplasmic reticulum and inositol 1, 4, 5-trisphosphate-induced Ca^{2+} release in mouse oocytes and embryos." Molecular biology of the cell **14**(1): 288-301.

Flach, G., M. Johnson, et al. (1982). "The transition from maternal to embryonic control in the 2-cell mouse embryo." The EMBO journal **1**(6): 681.

Gibbons, R. J., S. Bachoo, et al. (1997). "Mutations in transcriptional regulator ATRX establish the functional significance of a PHD-like domain." Nature genetics **17**(2): 146.

Griffiths AJF, M. J., Suzuki DT, et al. (2000). An Introduction to Genetic Analysis. New York, W. H. Freeman.

Haffray, P., A. Fostier, et al. (1995). "Influence du maintien en mer ou de la période de transfert en eau douce des reproducteurs de saumon atlantique *Salmo salar* sur la maturation sexuelle et la qualité des gamètes." Aquatic Living Resources **8**(02): 135-145.

Hamatani, T., M. G. Carter, et al. (2004). "Dynamics of global gene expression changes during mouse preimplantation development." Developmental cell **6**(1): 117-131.

Horvath, A. and B. Urbányi (2000). "The effect of cryoprotectants on the motility and fertilizing capacity of cryopreserved African catfish *Clarias gariepinus* (Burchell 1822) sperm." Aquaculture Research **31**(3): 317-324.

Howley, C. and R. K. Ho (2000). "mRNA localization patterns in zebrafish oocytes." Mechanisms of development **92**(2): 305-309.

Hu, J., F. Wang, et al. (2010). "Mouse ZAR1 - like (XM_359149) colocalizes with mRNA processing components and its dominant - negative mutant caused two - cell - stage embryonic arrest." Developmental dynamics **239**(2): 407-424.

Izquierdo, M., H. Fernandez-Palacios, et al. (2001). "Effect of broodstock nutrition on reproductive performance of fish." Aquaculture **197**(1-4): 25-42.

Kane, D. A. and C. B. Kimmel (1993). "The zebrafish midblastula transition." Development **119**(2): 447-456.

Keady, B. T., P. Kuo, et al. (2007). "MAPK interacts with XGef and is required for CPEB activation during meiosis in *Xenopus* oocytes." Journal of Cell Science **120**(6): 1093-1103.

Kishimoto, T. (2003). "Cell-cycle control during meiotic maturation." Current opinion in cell biology **15**(6): 654-663.

Kjørsvik, E., K. Hoehne-Reitan, et al. (2003). "Egg and larval quality criteria as predictive measures for juvenile production in turbot (*Scophthalmus maximus* L.)." Aquaculture **227**(1-4): 9-20.

Kjørsvik, E., A. Mangor-Jensen, et al. (1990). "Egg quality in fishes." Advances in Marine biology **26**: 71-113.

Kopeika, J., E. Kopeika, et al. (2003). "Detrimental effects of cryopreservation of loach (*Misgurnus fossilis*) sperm on subsequent embryo development are reversed by incubating fertilised eggs in caffeine." Cryobiology **46**(1): 43-52.

Kopeika, J., E. Kopeika, et al. (2004). "Effect of DNA repair inhibitor (3-aminobenzamide) on genetic stability of loach (*Misgurnus fossilis*) embryos derived from cryopreserved sperm." Theriogenology **61**(9): 1661-1673.

Kuranda, M. J. and N. Aronson (1986). "A di-N-acetylchitobiase activity is involved in the lysosomal catabolism of asparagine-linked glycoproteins in rat liver." Journal of Biological Chemistry **261**(13): 5803.

Labbe, C., A. Martoriati, et al. (2001). "Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout." Molecular reproduction and development **60**(3): 397-404.

Lahnsteiner, F. (2000). "Morphological, physiological and biochemical parameters characterizing the over-ripening of rainbow trout eggs." Fish Physiology and Biochemistry **23**(2): 107-118.

Latham, K. E. (1999). "Mechanisms and control of embryonic genome activation in mammalian embryos." International review of cytology **193**: 71-124.

Latham, K. E. and R. M. Schultz (2001). "Embryonic genome activation." Front Biosci **6**: D748-759.

Leray, C., G. Nonnotte, et al. (1985). "Incidence of (n-3) essential fatty acid deficiency on trout reproductive processes." Reproduction, nutrition, development **25**(3): 567.

Ma, J., F. Zeng, et al. (2006). "Basonuclin: a novel mammalian maternal-effect gene." Development **133**(10): 2053-2062.

Malcuit, C., M. Kurokawa, et al. (2006). "Calcium oscillations and mammalian egg activation." Journal of cellular physiology **206**(3): 565-573.

Manyu Li, H. F., Chao Tong, Dayuan Chen, Guoliang Xia and Qingyuan Sun (2002). "MAPK regulates cell cycle progression in pig oocytes and fertilized eggs." CHINESE SCIENCE BULLETIN **47**: 4.

McLay, D. W. and H. J. Clarke (2003). "Remodelling the paternal chromatin at fertilization in mammals." Reproduction **125**(5): 625-633.

Mendez, R., D. Barnard, et al. (2002). "Differential mRNA translation and meiotic progression require Cdc2-mediated CPEB destruction." The EMBO journal **21**(7): 1833-1844.

Michailidis, G., A. Argiriou, et al. (2010). "Expression of chicken zygote arrest 1 (Zar1) and Zar1-like genes during sexual maturation and embryogenesis." Veterinary research communications **34**(2): 173-184.

Michailidis, G., A. Kalivas, et al. (2009). "Expression and characterisation of the maternal factor Zygote arrest 1 (Zar1) gene in chicken tissues and embryos." Journal of Biological Research **11**: 21-27.

Mommsen, T., Korsgaard, B., (2008). Vitellogenesis. Fish Reproduction Science, Rocha, M.J., Arukwe, A., Kapoor, B.G. (Eds.): 1-36.

Nagler, J. (2000). "In vivo treatment with cycloheximide or actinomycin D inhibits early embryonic development in rainbow trout (*Oncorhynchus mykiss*)." Fish Physiology and Biochemistry **22**(1): 61-66.

Ourth, D. D., M. B. Narra, et al. (2007). "Comparative study of mannose-binding C-type lectin isolated from channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*)." Fish & Shellfish Immunology **23**(6): 1152-1160.

Palace, V. P. and J. Werner (2006). "Vitamins A and E in the maternal diet influence egg quality and early life stage development in fish: a review." Scientia Marina **70**(S2): 41-57.

Pankhurst, N., G. Purser, et al. (1996). "Effect of holding temperature on ovulation, egg fertility, plasma levels of reproductive hormones and in vitro ovarian steroidogenesis in the rainbow trout *Oncorhynchus mykiss*." Aquaculture **146**(3-4): 277-290.

Patiño, R. and C. V. Sullivan (2002). "Ovarian follicle growth, maturation, and ovulation in teleost fish." Fish Physiology and Biochemistry **26**(1): 57-70.

Payer, B., M. Saitou, et al. (2003). "< i> stella</i> Is a Maternal Effect Gene Required for Normal Early Development in Mice." Current Biology **13**(23): 2110-2117.

Pelegri, F. (2003). "Maternal factors in zebrafish development." Developmental dynamics **228**(3): 535-554.

Penetier, S., S. Uzbekova, et al. (2004). "Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15, and VASA in adult bovine tissues, oocytes, and preimplantation embryos." Biology of Reproduction **71**(4): 1359-1366.

Rime, H., N. Guitton, et al. (2004). "Post-ovulatory ageing and egg quality: a proteomic analysis of rainbow trout coelomic fluid." Reproductive Biology and Endocrinology **2**(1): 26.

Sakai, K., Nomura, M., Takashima, F. (1985). "Characteristics of naturally spawned eggs of red sea bream." Bull. Jpn. Soc. Sci. Fish **51**: 1395-1399.

Sangiorgio, L., B. Strumbo, et al. (2008). "A putative protein structurally related to zygote arrest 1 (Zar1), Zar1-like, is encoded by a novel gene conserved in the vertebrate lineage." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **150**(2): 233-239.

Schultz, R. M. (2002). "The molecular foundations of the maternal to zygotic transition in the preimplantation embryo." Human Reproduction Update **8**(4): 323-331.

Shields, R., N. Brown, et al. (1997). "Blastomere morphology as a predictive measure of fish egg viability." Aquaculture **155**(1-4): 1-12.

Stitzel, M. L. and G. Seydoux (2007). "Regulation of the oocyte-to-zygote transition." Science's STKE **316**(5823): 407.

Stoddard, J., J. Parsons, et al. (2006). "Early onset of embryonic mortality in sub-fertile families of rainbow trout (*Oncorhynchus mykiss*)." Reproduction, Fertility and Development **17**(8): 785-790.

Tadros, W. and H. D. Lipshitz (2005). "Setting the stage for development: mRNA translation and stability during oocyte maturation and egg activation in *Drosophila*." Developmental dynamics **232**(3): 593-608.

Tata, J. R. (1986). "Coordinated assembly of the developing egg." BioEssays **4**(5): 197-201.

Thorgaard, G. H., G. S. Bailey, et al. (2002). "Status and opportunities for genomics research with rainbow trout." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **133**(4): 609-646.

Tong, Z. B., C. A. Bondy, et al. (2002). "A human homologue of mouse Mater, a maternal effect gene essential for early embryonic development." Human Reproduction **17**(4): 903-911.

Tong, Z. B., L. Gold, et al. (2000). "Mater, a maternal effect gene required for early embryonic development in mice." Nature genetics **26**(3): 267.

Tong, Z. B. and L. M. Nelson (1999). "A mouse gene encoding an oocyte antigen associated with autoimmune premature ovarian failure." Endocrinology **140**(8): 3720-3726.

Uzbekova, S., M. Roy-Sabau, et al. (2006). "Zygote arrest 1 gene in pig, cattle and human: evidence of different transcript variants in male and female germ cells." Reprod Biol Endocrinol **4**: 12.

Vasudevan, S., E. Seli, et al. (2006). "Metazoan oocyte and early embryo development program: a progression through translation regulatory cascades." Genes & development **20**(2): 138.

Voronina, E. and G. M. Wessel (2003). "The regulation of oocyte maturation." Current Topics in Developmental Biology **58**: 53-110.

Wallace, R. A. and K. Selman (1981). "Cellular and dynamic aspects of oocyte growth in teleosts." American Zoologist: 325-343.

Wallace, R. A. and K. Selman (1985). "Major protein changes during vitellogenesis and maturation of *Fundulus* oocytes." Developmental biology **110**(2): 492-498.

Wang, Q. T., K. Piotrowska, et al. (2004). "A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo." Developmental cell **6**(1): 133-144.

Weigel, K. A. (2006). "Prospects for improving reproductive performance through genetic selection." Animal reproduction science **96**(3): 323-330.

Wishart, M. J. and J. E. Dixon (1998). "Gathering STYX: phosphatase-like form predicts functions for unique protein-interaction domains." Trends in Biochemical Sciences **23**(8): 301-306.

Wishart, M. J. and J. E. Dixon (2002). "The archetype STYX/dead-phosphatase complexes with a spermatid mRNA-binding protein and is essential for normal sperm production." Proceedings of the National Academy of Sciences **99**(4): 2112.

Wu, X., M. M. Viveiros, et al. (2003). "Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition." Nature genetics **33**(2): 187-191.

Wu, X., P. Wang, et al. (2003). "Zygote arrest 1 (Zar1) is an evolutionarily conserved gene expressed in vertebrate ovaries." Biology of Reproduction **69**(3): 861-867.